# ESTABLISHMENT AND REVERSAL OF HIV-1 LATENCY IN PRIMARY CD4+ NAIVE AND CENTRAL MEMORY T CELLS

by

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**Establishment and Reversal of HIV-1 Latency in Primary CD4+ Naïve and Central Memory T Cells**

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University of Pittsburgh, 2016

Human immunodeficiency virus type 1 (HIV-1) persists as a lifelong infection, due to the establishment of a latent viral reservoir in rCD4+ T cells, representing a major barrier to eradication of HIV-1 infection. Therapeutic approaches to eliminate this latent reservoir have included the "kick and kill" strategy, which involves the administration of a latency reversing agent (LRA) to "kick" HIV-1 out of latency and "kill" the HIV-1-infected cells by cytolytic or viral cytopathic effects. rCD4+ T cells are heterogeneous, consisting of naïve ($T_N$), stem cell-like memory ($T_{SCM}$), central memory ($T_{CM}$), transitional memory ($T_{TM}$), effector memory ($T_{EM}$), and terminally differentiated ($T_{TD}$) cells. Preliminary studies demonstrated that the $T_{CM}$ and $T_{TM}$ cell subsets constitute the major proportion of the latent HIV-1 reservoir in infected individuals on ART, while HIV-1 DNA is consistently lower in $T_N$ cells. Consequently, there has been little emphasis on studying HIV-1 latency in $T_N$ cells. The primary goal of this thesis was to understand latent HIV-1 infection in $T_N$ cells in comparison to $T_{CM}$ cells, through the development of an appropriate in vitro primary cell model of HIV-1 latency in $T_N$ and $T_{CM}$ cells, as well as in ex vivo patient-derived cells. Employing the "kick and kill" approach to $T_N$ and $T_{CM}$ cells in our in vitro primary cell model revealed that although $T_N$ cells contained significantly less HIV-1 DNA than $T_{CM}$ cells, following reactivation, they produced as much, if not more, virus than $T_{CM}$ cells when normalized for infection frequency. This finding was also observed using ex vivo cells from 4 of 7 donors. Furthermore, we found that similar levels of replication-

competent virus were recovered from $T_N$ and $T_{CM}$ cells when corrected for infection frequency. These findings suggest that quantifying HIV-1 DNA alone may not be predictive of the size of the inducible latent reservoir in different CD4+ T cell subsets. Furthermore, although $T_N$ cells constitute only a fraction of the HIV-1 DNA reservoir, they may contribute significantly to viral rebound following treatment interruption or failure. Thus, a greater attention should be given to the latent viral reservoir in $T_N$ cells in HIV-1-infected individuals on ART.

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# PREFACE

First and foremost, I would like to thank my mentor, Nic, for always pushing me, often a little too hard, to be the greatest student and researcher I could be. One of my favorite things about working for Nic was his hands-off approach, which ultimately demanded that I become an independent worker and learn how to develop and implement many of my own ideas. This has taught me the invaluable lessons of independent thought and critical thinking. It has also been a great pleasure to give Nic as hard a time as possible, making work as entertaining as it can be.

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# 1.0 INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) is the causative agent of acquired immunodeficiency syndrome (AIDS). HIV-1 infection has been one of the most devastating plagues of modern times and it is currently the second leading killer among infectious diseases worldwide, following tuberculosis[13, 14]. As of 2015, there were nearly 37 million people living with HIV-1, and of those 37 million, 17 million were receiving antiretroviral therapy to treat infection[15].

The HIV/AIDS epidemic was first brought to light in 1981 by a case report describing *Pneumocystis carinii* pneumonia (PCP) in five homosexual men with no known underlying illness or immunodeficiency[16]. This was of great concern because PCP in the United States had previously only been associated with severely immune-compromised individuals[17]. Shortly after this initial report, several other isolated case reports from around the United States described similar pathologies in previously healthy homosexual men, along with the addition of Kaposi's sarcoma, mucosal candidiasis, and other opportunistic infections[18-28].

Many of the initial reports speculated that the causative agent for this unknown immunodeficiency was cytomegalovirus (CMV), which was found in nearly all of the affected individuals. It wasn't until 1983 that Barre-Sinoussi et al. from the Pasteur Institute in France first demonstrated that a retrovirus closely related to the recently identified human T cell leukemia viruses (HTLV-I and HTLV-II) was associated with AIDS[29]. The direct proof that

HIV-1 (initially named lymphadenopathy virus [LAV] by the French or HTLV-III by the US) was the causative agent of AIDS came in four publications the following year from Dr. Robert Gallo's lab at the National Cancer Institute (NCI)[30-33].

There are two different types of HIV, type 1 and type 2, transmitted to humans through several different zoonotic events[34]. HIV-1 was transmitted through chimpanzees[35] and gorillas[36, 37], and HIV-2 was transmitted through sooty mangabeys[38-40]. Each zoonotic event resulted in a new group. For HIV-1, there are four groups. Group M, which is the main group and pandemic form of HIV-1, can further be subdivided into at least nine different subtypes: A, B, C, D, F, G, H, J, K, as well as circulating recombinant forms. Additionally, there are groups N[41], O[42, 43], and P[36]. These last three groups are rare and are largely restricted to Western Africa[44]. HIV-2 is less pathogenic than HIV-1 and typically progresses much more slowly to AIDS[45]. HIV-2, largely confined to West Africa, is also less transmissible and is in decline[44]. The work presented in this thesis will focus solely on HIV-1, group M, subtype B.

## 1.1 HUMAN IMMUNODEFICIENCY VIRUS TYPE-1 AND THE AIDS EPIDEMIC

### 1.1.1 HIV-1 pathogenesis and disease

In the two years between the first description of AIDS[16] and the first isolation of the virus thought to cause the disease[29], a remarkable wealth of information about risk factors associated with infection, modes of transmission, and clinical manifestations of disease was characterized. It was determined that HIV-1 was spread through intravenous drug use, sexual contact, blood

transfusions, organ transplantation, and from mother-to-child[46-54]. The biggest risk factor for contracting HIV-1 was to be a man who had unprotected sex with men.

HIV-1 infection and the pathogenesis of AIDS can loosely be broken down into three stages: (i) primary or acute infection, (ii) clinical latency, (ii) and the development of AIDS. In the early 1980s, people went undiagnosed with HIV-1 until they presented with AIDS, largely because the etiological agent had not yet been identified and appropriate diagnostic tests had not yet been developed to detect the virus. During acute HIV-1 infection, there is a short eclipse phase that lasts from 1-2 weeks were the virus replicates uncontrollably and spreads from the initial site of infection to other tissues and organs. This is followed by what is called "acute retroviral syndrome" in which 50-70% of patients typically present with severe flu-like symptoms, including fever, lymphadenopathy, joint pain, rash, headaches, and a sore throat[55, 56]. Because of these non-specific symptoms, most cases at this stage went undiagnosed or were misdiagnosed as a non-specific viral infection[55, 57]. This phase typically lasts from two weeks to two months, and is characterized by a rapid decline in CD4+ T cells[58], as well as having very high levels of HIV-1 in the blood, often reaching or exceeding $10^7$ copies/mL[56, 59-61]. This spike in viremia, often referred to as 'peak viremia', is reached just before the host's HIV-1-specific adaptive immune response appears and is able to recognize viral antigens and destroy free virus or HIV-1-infected cells[62-64].

The period of clinical latency is misleading because the virus is still replicating within the host and causes lymphoid tissue destruction. By definition, during clinical latency, an infected individual does not present with any signs or symptoms of disease, and may not even know that they are infected. During this period, which typically lasts from 1-10 years, HIV-1 replication is maintained at a viral set-point, in which plasma viremia remains stable, typically between 10,000

– 100,000 copies/mL of plasma, the peripheral CD4+ T cell count continuously declines and immune function gradually deteriorates[65]. Throughout infection, there is also ongoing chronic immune activation, which among other things, results in irreversible collagen deposition and fibrosis of secondary lymphoid tissues[66-70]. This results in impaired thymopoiesis and lymphopoiesis, preventing the generation and maturation of new lymphocytes.

At the point when CD4+ T cell counts decline to 200 cells/mm$^3$ or less, a person is diagnosed with AIDS. With diminishing CD4+ T cell counts, accompanied with immune dysfunction and dysregulation, the immune system is no longer able to contain the infection and opportunistic infections appear. In the absence of therapeutic intervention, the mortality rate following AIDS diagnosis is >95% within 5 years with a median survival time of only 1 year[71].

### 1.1.2 HIV-1 replication

The HIV-1 replication cycle can loosely be broken down into seven steps: (1) entry, (2) reverse transcription, (3) integration, (4) transcription/translation, (5) assembly, (6) budding, and (7) maturation. It was not long after the discovery of HIV-1 that it was determined that HIV-1 primarily infects CD4+ T cells[72] and that CD4 was the major receptor required for viral entry[73, 74]. It wasn't until 1996, however, that the two main co-receptors, CCR5[75] and CXCR4[76], were identified. Which co-receptor the virus uses for cellular entry determines the tropism of the virus and they are termed either R5- or X4-tropic[77]. Some viruses are able to use both CCR5 and CXCR4 as co-receptors and these viruses are termed dual-tropic[77].

HIV-1 entry is initiated when the HIV-1 gp120 subunit of the envelope (Env) protein binds to CD4 on the surface of a cell[78, 79]. This initial interaction causes a conformational change in Env that allows for subsequent co-receptor binding. Co-receptor binding initiates membrane

fusion in which the fusion peptide of the gp41 subunit of Env inserts into the cell membrane[80]. Membrane fusion is completed following six-helix bundle formation between the viral membrane and the cellular membrane[81, 82]. Following fusion of the HIV-1 virion to a target cell, a fusion pore is formed[83] and the viral capsid core is released into the cytoplasm[84]. The entering capsid contains all of the viral components necessary for HIV-1 replication, including two copies of the positive sense single-stranded viral RNA genome, as well as the three HIV-1 enzymes: reverse transcriptase (RT), integrase (IN), and protease (PR). In addition to containing viral components necessary for HIV-1 replication, the incoming capsid also contains a few cellular factors, including $tRNA^{Lys3}$ and cellular dNTPs, both of which are required for the initiation of reverse transcription (see below).

Once in the cytoplasm, RT converts the single-stranded RNA genome into double stranded DNA in a process known as reverse transcription (reviewed in [85, 86]). Although this process can initiate prior to cell entry, reverse transcription cannot complete without access to cellular dNTPs in the target cell[87]. Following reverse transcription, the viral genome is imported into the nucleus in association with both host and viral proteins, including IN[88]. Integration of the viral genome is mediated by HIV-1 IN in a non-random manner[89]. Once the HIV-1 genome is integrated into the host genome, it is termed a provirus[90].

Following integration, the resulting provirus is susceptible to transcription of its genome. HIV-1 transcription occurs in three stages. The HIV-1 genome contains both an identical 5' and 3' long terminal repeat (LTR) sequence, both of which can act as the viral promoter, although the 5' LTR is highly favored over the 3' LTR[91] (Fig. 1). In early infection, only low levels of completely spliced HIV-1 mRNAs are produced that encode the three regulatory proteins, the trans-activator of transcription (Tat), Rev, and Nef (Fig. 1). With increases in Tat, HIV-1

transcription dramatically increases, resulting in incompletely spliced mRNAs that encode the viral envelope glycoprotein, Env, and the accessory proteins Vif, Vpr, and Vpu (Fig. 1). The last mRNA transcripts to be produced are the full-length unspliced transcripts that serve as both the virion genomic RNA and the mRNA for the Gag and Gag-Pol polyproteins[92, 93] (Fig. 1). Tat increases HIV-1 transcription through interaction with the HIV-1 transactivation response (TAR) element, a highly conserved RNA structure that forms at the 5' end of nascent viral transcripts, resulting in processive transcription elongation. While completely spliced HIV-1 mRNAs are exported to the nucleus through normal cellular pathways, incompletely and unspliced HIV-1 mRNAs require Rev for export from the nucleus to the cytoplasm, through direct interactions with the Rev response element (RRE) RNA target sequence[12] (Fig. 1).

HIV-1 mRNAs are exported to the cytoplasm through the nuclear pore complex (NPC), where protein translation can occur through normal cellular pathways. Env and the accessory protein Vpu are translated on the rough endoplasmic reticulum (ER), whereas Gag, Gag-Pol, and other accessory proteins are translated on cytosolic polysomes[94]. Following translation, the Env glycoproteins are transported to the plasma membrane via the cellular secretory pathway[95]. The Gag and Gag-Pol polyproteins are translated in the cytoplasm as protein precursors (Fig. 1). Both the Gag and Gag-Pol polyproteins contain the structural proteins matrix (MA, p17), capsid (CA, p24), and nucleocapsid (NC, p7) (Fig. 1). The Gag-Pol polyprotein also contains the viral enzymes, RT, IN, and PR (Fig. 1). Both the Gag and Gag-Pol polyproteins are targeted to the plasma membrane by the MA domain, which subsequently promotes incorporation of Env glycoproteins to the forming virion[95-97]. The CA domain drives Gag multimerization at the plasma membrane while the NC domain recruits the viral genomic RNA into the forming virion[95, 98].

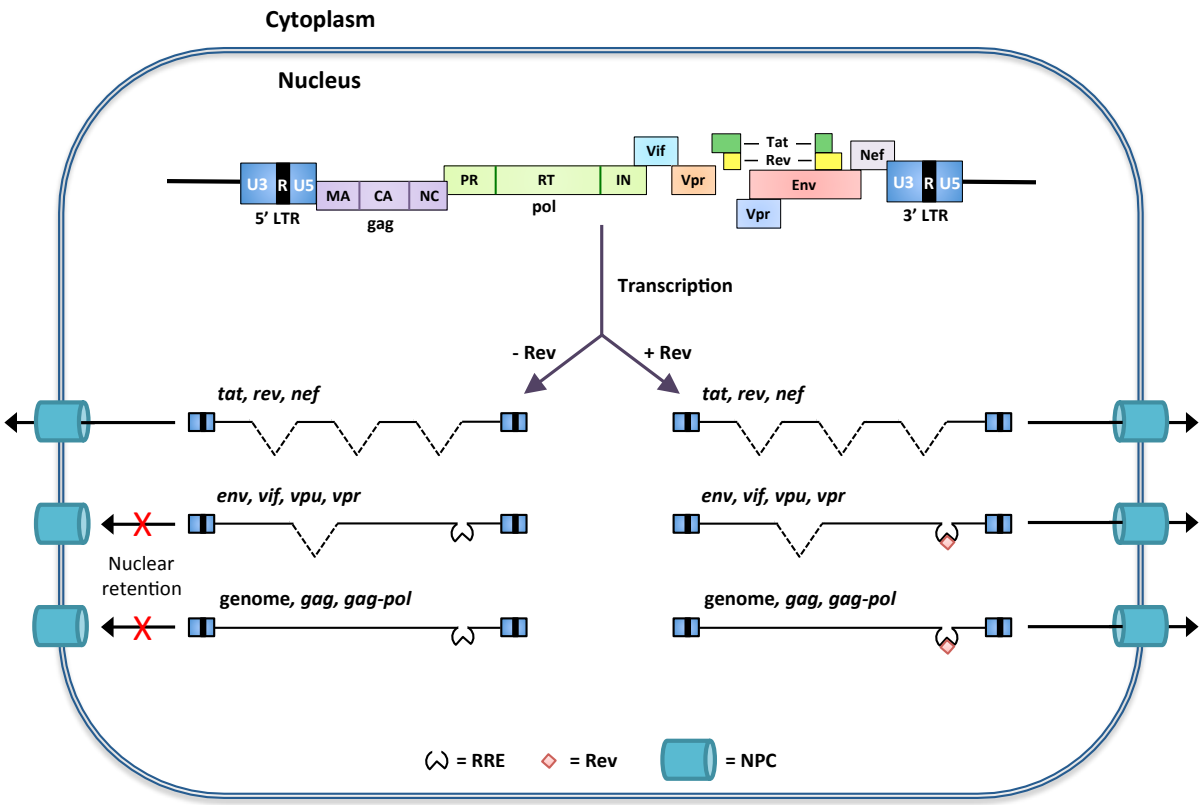



**Figure 1: HIV-1 genome organization, transcription, and Rev-mediated mRNA export.** A simplified overview of the HIV-1 genome organization, transcription of major mRNA species, and nuclear export of different mRNAs are shown. Upon transcriptional activation, different HIV-1 mRNAs are produced, including completely spliced, incompletely spliced, and unspliced species. Completely spliced mRNAs are able to export the nucleus and give rise to Tat, Rev, and Nef. In the absence of Rev, incompletely spliced and unspliced mRNAs are retained in the nucleus. In the presence of Rev, incompletely and unspliced mRNAs are able to export the nucleus to the cytoplasm, through direct interactions with Rev and the Rev response element (RRE) RNA target sequence. NPC = nuclear pore complex. (Figure adapted from Cullen, 2003 [doi:10.1016/S0968-0004(03)00142-7][12], with permission from *Elsevier* ©).

Following virion assembly at the plasma membrane, budding is mediated by endosomal sorting complex required for transport (ESCRT) proteins, which catalyze membrane fission[99]. The resulting virion is in an immature form and is non-infectious without further processing. Virion maturation occurs following activation of PR, in which PR then cleaves the Gag and Gag-Pol polyprotein precursors into their mature protein forms. These cleavage events result in morphological structural changes that lead to the formation of a mature, infectious virion.

### 1.1.3 The development of antiretroviral therapy and combination antiretroviral therapy: turning a death sentence into a chronic illness

With the identification of HIV-1 as a novel human retrovirus that causes severe and rapid disease, ultimately resulting in death, the race to develop or identify therapeutics to treat infection and prevent disease progression became a top priority in biomedical research. Antiretroviral drugs target five steps in the HIV-1 replication cycle: chemokine co-receptor binding, fusion, reverse transcription, integration, and maturation. Current Food and Drug Administration (FDA)-approved drugs fall into six classes based on the interaction or viral enzyme they inhibit: nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), integrase strand-transfer inhibitors (InSTIs), fusion/entry inhibitors, and co-receptor antagonists.

In the early 1980s, an AIDS diagnosis was essentially a death sentence. Following diagnosis, the median survival time was just over one year[71]. In 1987, the first antiretroviral drug to treat HIV-1 was FDA-approved. This drug was 3′-azido-3′-dideoxythymidine (azidothymidine, zidovudine, AZT), a chain-terminating thymidine nucleoside analog belonging to the class of NRTIs. NRTIs block HIV-1 replication by acting as chain terminators during reverse transcription. These drugs lack a 3’-hydroxyl group at the sugar moiety, which prevents the formation of a 3’-5’-phosphodiester bond between the NRTI and the incoming 5’-nucleoside triphosphate, resulting in the termination of viral DNA synthesis. Early clinical studies found that AZT treatment resulted in short-term increases in CD4+ T cell counts and improved immunological function, as well as decreases in opportunistic infections[100-104]. Follow-up studies on the long-term use of AZT, however, quickly revealed that AZT monotherapy provided limited benefit to HIV-1-infected individuals, delaying disease progression by only 1-3 years, with no

overall change in survival[105-110]. These results were likely due to the rapid appearance of drug-resistant HIV-1 following initiation of therapy, in addition to AZT-related toxicities[111-114].

The development of NRTIs rapidly expanded, with several additional drugs reaching FDA-approval in the early 1990s[115-118]. Studies conducted using NRTIs as monotherapy or in combination as dualtherapy quickly shed light on the complex nature of HIV-1 infection[119-122]. Combinations of two NNRTIs showed slight delays in disease progression compared to monotherapy alone[119-122]. However, it quickly became clear that drug resistance to one NRTI could confer resistance to another NRTI[123-125]. These findings suggested that while scientists and physicians were on the correct path of using drug combinations to treat HIV-1, additional drug targets would need to be included in the treatment of HIV-1 to obtain long-term suppression and prevent outgrowth of drug-resistant variants.

In an HIV-1-infected individual, there are typically between $10^4$-$10^5$ virions/mL of plasma, with a turnover rate of approximately $10^{10}$ virions per day[1, 2, 126]. Combined with the low fidelity of HIV-1 RT, which makes about 1-10 mutations per cDNA synthesis[127, 128], it was not surprising that monotherapy treatment led to the rapid expansion of drug resistant mutants[109, 129, 130]. Based on these characteristics, it was predicted that a viral quasispecies with reduced susceptibility to one or two drug classes would likely exist in an individual before initiation of therapy[131]. Mathematical modeling predicted that any combination of antiretroviral drugs (ARVs) that required at least three mutations to generate resistance would provide durable suppression of viral replication[131-134]. These analyses explained the limited and fading effects of mono or dual therapy, ultimately providing the impetus for triple combination therapy.

In the mid 1990s, several additional ARVs became FDA-approved and included two new drugs classes: the NNRTIs[135], and the PIs[136, 137]. NNRTIs differ from NRTIs in that they are

allosteric inhibitors of RT. NNRTIs bind to a hydrophobic pocket proximal to the RT active site[138], inducing a conformational change in the substrate-binding site that inhibits polymerase activity[138, 139]. PIs act as competitive inhibitors of HIV-1 PR by binding to the active site, thereby preventing cleavage of viral polyprotein precursors and inhibiting virion maturation[140]. With the increase in ARVs and drug classes, treatment of HIV-1-infected individuals with triple drug combinations was quickly underway. Triple drug combinations were able to improve both long-term immunological and virologic outcomes while maintaining viral suppression[135, 141, 142]. The clinical benefits demonstrated in these studies ultimately led to the FDA approval of triple combination antiretroviral therapy (termed ART from here on) in 1996.

With the ongoing need to develop less toxic and more efficacious ARVs, three additional drug classes and many additional ARVs were developed and FDA-approved in the 2000s. In 2003, the first and only fusion inhibitor was approved, enfuvirtide, which acts as a peptide mimetic of a portion of gp41, preventing virion fusion to the target cell membrane[143, 144]. In 2007, the first and only co-receptor antagonist, maraviroc (MVC), which is a CCR5 antagonist, was approved, along with the first InSTI, raltegravir (RAL). MVC is an allosteric inhibitor that alters the extracellular conformation of CCR5, preventing recognition by gp120[145, 146]. InSTIs target the active site of IN and block the reaction of HIV-1 DNA strand transfer into the cellular DNA genome, the process of integration[147-150].

To date, there have been over 30 FDA-approved antiretroviral drugs, including five single tablet combinations. The development of ART has revolutionized modern medicine. Through continued development of more potent ARVs and ARV combinations, individuals with HIV-1 who are on ART now have virtually the same life expectancy as those who are uninfected[151]. Although the number of people living with HIV-1 has been steadily increasing

over time, reaching approximately 37 million individuals in 2015, there has been a significant decline in HIV-1-related morbidity and mortality thanks to the tremendous advances in ART[152]. Not only have ART regimens improved over time, there has been an incredible global effort to increase access to ART, increasing coverage from 3% to 46% over the last 15 years world-wide[15]. Ongoing efforts are being made to improve ART regimens through the development of novel ARVs, as well as efforts to increase HIV-1 diagnosis and treatment to decrease HIV-1 transmission and increase clinical outcomes following diagnosis[15]. The fight against HIV/AIDS is far from over, but the expanding global efforts and increasing collaborations are putting the end of this epidemic within reach.

## 1.2 VIRAL PERSISTENCE WHILE ON THERAPY REVEALS A LONG-LIVED LATENT RESERVOIR

ART is now the standard care of treatment, and where available, administered immediately following diagnosis. ART blocks viral replication and reduces plasma viremia to below the limit of detection of clinical assays (50 copies/mL of plasma)[153]; however, it is not curative. In 1995, work by Chun et al. demonstrated for the first time that resting CD4+ T cells (rCD4) isolated from HIV-1-infected individuals contained integrated HIV-1 DNA and could thus function as a latent reservoir[154]. Furthermore, they demonstrated that these latently infected cells did not accumulate over time, suggesting that productively infected CD4+ T cells do not generally survive long enough in vivo to return to a resting memory state. In 1997, only a year after the introduction of ART, three independent groups identified the presence of an inducible latent viral reservoir in HIV-1-infected individuals on ART[155-157]. Importantly, the inducible virus recovered

from this reservoir was also found to contain replication-competent virus. It was also demonstrated that the latent viral reservoir resided in rCD4 T cells[155]. (Further discussion of the composition of the latent reservoir will be discussed in section 1.4.1.) Detailed quantitative analyses estimated that the frequency of rCD4 T cells harboring integrated HIV-1 DNA was on the order of 1-10 cells per million rCD4 T cells, with a total body load of $10^6$–$10^7$ latently infected cells[155, 158]. Of these latently infected cells, approximately 1% were shown to contain inducible, replication-competent virus[155, 159].

Early observations of the decay kinetics of plasma HIV-1 RNA revealed that it declines in two distinct phases: an initial rapid phase with a half-life of 1-2 days, likely due to the rapid elimination of cell-free virions, followed by a shorter second phase, with a half-life of 1-4 weeks, likely due to the loss of productively infected cells[3] (a summary of HIV-1 RNA and DNA decay following ART is shown in Fig. 2). Mathematical modeling based on these early decay characteristics estimated that ART could eliminate HIV-1-infected cells in an individual in 2-3 years[3]. Based on the available information at the time, this provided hope for a cure in the near future. This prediction, however, was quickly proven to be incorrect.

In 1999, a longitudinal quantitative evaluation of latently infected cells in HIV-1-infected individuals on ART estimated the half-life of latently infected rCD4 T cells to be 44 months, indicating that it would take >60 years of ART to eradicate infection, assuming a total body reservoir of only $10^5$ cells[159]. This finding was confirmed in 2003 by a larger study of HIV-1-infected individuals who had been on suppressive ART for up to 7 years[4]. More recent DNA analyses of HIV-1-infected individuals who have been on ART for 7-12 years, revealed that following ART, there is an initial rapid decline in HIV-1 DNA over the first year of approximately 85%, followed by a slower decay of approximately 23%/year between years 1-4,

while there is no measurable decay thereafter[7]. This indicates that long-term ART is unable to eliminate the pool of latently infected cell, even after up to 12 years of therapy.

In 2003, a quantitative reverse transcriptase PCR (qRT-PCR) single copy assay was developed that could detect down to a single copy of HIV-1 RNA in 1mL of plasma[160]. This study demonstrated that despite maintaining suppressive ART, low-level viremia persisted in all subjects evaluated. A follow-up study in 2007 found that despite long-term suppression, ultrasensitive PCR methods were able to detect low-level residual viremia in approximately 80% of patients who were on ART for at least 7 years[6]. This study also showed that low-level plasma viremia persists at a median set point of approximately 3 copies/mL for at least 7 years, identifying a third phase of decay. It wasn't until just this year that yet a fourth phase of HIV-1 RNA decay was identified as having a half-life of approximately 11.5 years[8]. This analysis was only made possible through larger scale longitudinal analyses of individuals on ART for greater periods of time than were previous possible to evaluate.

Although the source of residual viremia while on ART still remains somewhat controversial, three major hypotheses have been proposed to explain this phenomenon: (i) limited ongoing cycles of replication in the presence of ART, (ii) production of HIV-1 from sanctuary sites where antiretroviral drugs do not attain maximal penetration, and (iii) sporadic release of virus from latently infected cells[4, 159, 161-165]. Extensive research has shown a lack of viral sequence evolution while on ART[166-177] and a lack of reduction in residual viremia or the size of the latent reservoir through the addition of a fourth or fifth ARV to treatment (treatment intensification)[166, 178-189], which suggests that the major source of residual viremia while on ART comes from transcription of viral RNA from stable latent reservoirs.

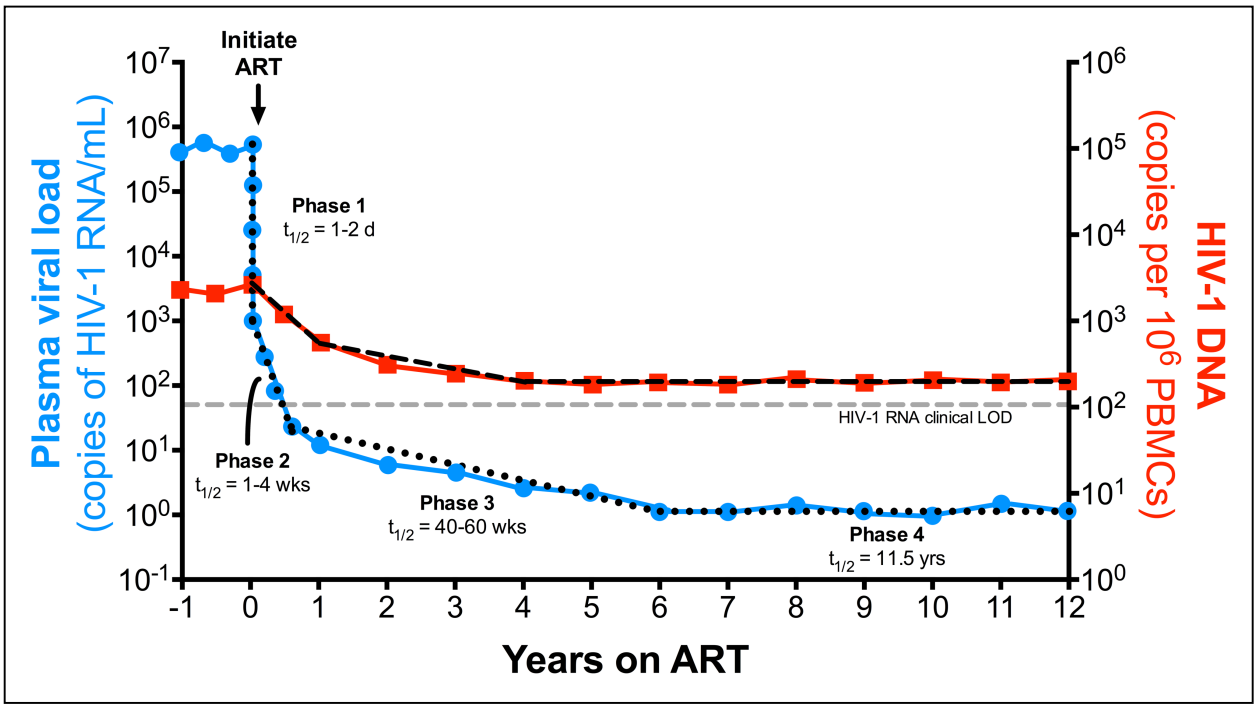



**Figure 2: Decay of HIV-1 RNA and DNA following initiation of ART.** Observed decay of plasma HIV-1 RNA (blue) and PBMCs containing HIV-1 DNA (red) following initiation of ART. Upon initiation of ART, plasma viremia declines rapidly in a matter of days to weeks, likely due to the loss of free virus and activated CD4+ T cells (Phase 1) followed by the loss of monocytes/macrophages, and partially activated CD4+ T cells (Phase 2). The third and fourth phases of decay are likely due to the turnover of long-lived reservoirs, such as naïve CD4+ T cells and memory CD4+ T cells. HIV-1-infected cells decay much slower, with an initial phase between years 0-1 following treatment initiation with a slope of -0.86. The second phase occurs between years 1-4 with a slope of -0.11. There is no significant decay of HIV-1-infected cells between years 4-12. d = days. wks = weeks. yrs = years. LOD = limit of detection. This figure is a generalized diagram generated using data combined from several studies[1-9], and is an updated version of the graph previously published by Hilldorfer et al, 2012[10].

Despite long-term suppression of HIV-1 replication by ART, treatment interruption studies have revealed that plasma HIV-1 RNA levels quickly rebound, typically within 1-8 weeks, reaching near pre-therapy levels[190-196]. The persistence and stability of this latent reservoir requires remaining on ART for life, which comes with its own challenges. Therapeutic strategies to eliminate the latent reservoir are rigorously under investigation with the hopes of achieving a cure.

## 1.3 THE ONE AND ONLY CURE: LESSONS LEARNED FROM TIMOTHY RAY BROWN AND OTHER STEM CELL APPROACHES TO CURE HIV-1 INFECTION

### 1.3.1 Early studies of transfusions, stem cell transplantations, and chemotherapy to treat HIV-1 infection

The beginning of the AIDS epidemic presented largely with homosexual, previously healthy men, who were now suffering from severe immunodeficiency, opportunistic infections, and many presented with cancers such as Kaposi's sarcoma or non-Hodgkin's lymphoma[20, 197, 198]. Because of this common association of AIDS with cancer, the HIV/AIDS and oncology fields have been closely linked since the beginning. Prior to ART, cancer was a significant cause of mortality in HIV-1-infected individuals, largely due to uncontrolled co-infections with oncogenic viruses such as Epstein-Barr virus, Kaposi's sarcoma-associated herpesvirus, and human papillomavirus[199]. Prior to ART, nearly 80% of malignancies in HIV-1-infected individuals were AIDS-related[200].

In the early 1980s, it had been shown that allogeneic stem cell transplantation (ASCT) was a successful clinical treatment option for children with severe combined immunodeficiency (SCID)[201]. Positive outcomes using ASCT to treat an immunodeficiency disorder laid the foundation for using ASCT in HIV-1-infected individuals to try to restore immune function and dampen disease progression[202]. Prior to ART, AIDS patients presented with such advanced disease that ASCT was limited due to the toxicity of the conditioning regimen required, including high-dose chemotherapy and radiation[203]. Therefore, early studies focused more on donor lymphocyte infusions, which do not require intensive conditioning regimens[204, 205]. These studies were unsuccessful, largely due to lack of engraftment and severity of disease prior to

infusion[202]. Additional studies conducted between 1983-1984, were performed using allogeneic stem cells from HLA-identical siblings or twins, which did not require a pre-transplantation conditioning regimen[206-209]. These studies also demonstrated little to no benefit in the treatment of AIDS, indicating that lymphocyte infusion or ASCT without conditioning is not beneficial to the clinical course of HIV-1 infection.

With FDA-approval of AZT in 1987, ASCT was again revisited with the hope that if viral suppression could be maintained during transplantation, donor cells could be protected from infection[202]. Several studies were conducted to test the added protection of AZT as well as pre-transplantation conditioning, including chemotherapy and total body irradiation (TBI), in HIV-1-infected individuals with underlying lymphoma or leukemia[210-212]. Much like the earlier studies using identical twins or siblings, syngeneic transplantations performed in the presence of AZT were unsuccessful and had no effect on HIV-1 infection. Individuals who underwent ASCT in the presence of AZT showed more promising results. Despite all individuals ultimately succumbing to illness shortly after transplantation from recurrent lymphoma, graft-versus-host disease (GVHD), or acute respiratory distress syndrome, they showed no evidence of viremia prior to death. In two individuals, post-mortem autopsy and extensive tissue sampling revealed no detectable HIV-1 RNA and little to no detectable HIV-1 DNA by conventional PCR methods used at the time[210, 212]. This led the authors to conclude that the combination of myeloablative chemotherapy and ASCT could eradicate HIV-1 from an individual while AZT protected new cells from becoming infected.

During this same time period, despite evidence of clinical benefit, several other groups performed syngeneic HSCT on a total of 20 HIV-1-infected individuals, comparing individuals who were treated with AZT compared to those who were untreated[213-217]. Although these studies

did show an increase in CD4+ T cell count following transplantation, there was persistence of HIV-1 in both groups, and there were no significant differences in disease progression or clinical outcome between the AZT-treated versus untreated arms.

Despite two rare cases that achieved undetectable HIV-1 following ASCT, these studies collectively demonstrated that stem cell transplantation, even with the addition of AZT, was unsuccessful at controlling viral replication. Most individuals died within the first year following transplantation, due to recurrent malignancy, GVHD, or infection, making long-term analyses impossible. These studies were further complicated by the underlying bone marrow toxicity associated with AZT[218] that may have contributed to poor engraftment and failure to achieve immune reconstitution following transplantation.

With the introduction of ART in 1996, clinical prognoses of patients with HIV-1 improved significantly[219]. ASCT could now be performed in the presence of ongoing ART. At this time, many of these studies were focused on clinical outcome and survival rather than eradication or reduction of HIV-1[220-226]. In addition to ASCT, studies were also being conducted using autologous HSCT in HIV-1-infected individuals on ART[227-230]. In general, these studies showed long-term survival and successful treatment of hematological malignancies. Many of these transplantations were also conducted using reduced-intensity conditioning (RIC), which limited toxicity and side effects from the procedure[231]. These studies demonstrated that HIV-1-related malignancies could now be successfully treated with HSCT without significant increases in transplant-related mortality.

Between 2007-2014, a handful of studies were also conducted to specifically look at the effect of autologous HSCT on viral persistence following transplantation[232-235]. Initial studies were met with conflicting results finding no changes in the level of HIV-1 DNA in peripheral

blood mononuclear cells (PBMCs) in some individuals but a reduction in HIV-1 DNA in PBMCs in others[232, 233]. Two follow up studies were conducted here at the University of Pittsburgh to specifically evaluate the effect of autologous HSCT versus chemotherapy alone, respectively, on viral persistence. The results from these two studies revealed that autologous HSCT did not eliminate or significantly reduce the size of the HIV-1 reservoir[235] and that moderate intensity chemotherapy given to HIV-1-infected individuals with HIV-1-associated malignancies had no long-term effect on plasma viremia or HIV-1 DNA in PBMCs[234].

Although it is clear that neither allogeneic nor autologous HSCT are a successful therapeutic option for eradicating or significantly reducing the HIV-1 reservoir, substantial advances have been made. Due to improved ART regimens and autologous HSCT procedures, there is currently no difference between HIV-1-infected and uninfected individuals in terms of clinical outcome and survival following autologous HSCT treatment for lymphoma[236-238]. In the presence of fully suppressive ART, both allogeneic and autologous HSCT are now a common practice to treat HIV-1-associated malignancies[239].

### 1.3.2 Timothy Ray Brown: the face of HIV-1 cure

Timothy Ray Brown, also known as the "Berlin Patient," is an American homosexual man who contracted HIV-1 in 1995 while in Barcelona, Spain[240]. He was immediately put on AZT following diagnosis and was switched to ART once it became available in 1996. After living a relatively normal, healthy life for the next 11 years[241], Mr. Brown was diagnosed with acute myeloid leukemia (AML) while studying in Berlin, Germany[242]. At the time of his AML diagnosis, Mr. Brown's CD4+ T cell count was 415 cells per $mm^3$, his viral load was undetectable, and he was otherwise healthy[242]. Mr. Brown underwent extensive chemotherapy to

treat his cancer[242], and despite initial successes with chemotherapy, the AML quickly returned. This relapse indicated that he now needed to undergo TBI and receive an ASCT[242].

In 1996, it had first been shown that individuals who were homozygous for the CCR5delta32 mutation were generally resistant to HIV-1 infection[243, 244]. Individuals who were heterozygous CCR5/CCR5delta32 were found to be less susceptible to HIV-1, and those who did become infected progressed more slowly to AIDS than wild type CCR5/CCR5 individuals[244-248]. The global frequency of individuals that are homozygous for CCR5delta32 is very low, being identified in only 10% of people of European descent and 2-5% of individuals throughout Europe, the Middle East and the Indian subcontinent[249]. The frequency of individuals who are heterozygous for CCR5delta32 is much higher, reaching close to 20% in some populations[250].

Given his knowledge on CCR5delta32 frequency and the resulting resistance to HIV-1 infection, Mr. Brown's oncologist investigated whether a CCR5delta32 homozygous donor for stem cell transplantation could be identified[242]. Interestingly, Mr. Brown had a heterozygous genotype for CCR5delta32[242]. However, it is unclear if this had any impact on his disease progression or clinical outcome. One HLA-identical, homozygous CCR5delta32 match was identified[242]. Mr. Brown underwent conditioning chemotherapy, TBI, and received prophylaxis against GVHD containing rabbit antithymocyte globulin[242], as well as cyclosporine and mycophenolate mofetil, both of which are immunosuppressive drugs that help prevent graft rejection following transplantation[251, 252]. He received ART until the day before transplantation, and engraftment was achieved 13 days after the procedure[242]. Complete chimerism was achieved 61 days after transplantation and on day 159 following transplantation, a rectal biopsy was negative for HIV-1 DNA[242].

Nearly eleven months after transplantation, Mr. Brown's AML relapsed and his chimerism dropped to 15%[242]. He then underwent reinduction therapy and TBI before receiving a second ASCT from the same CCR5delta32 homozygous donor[242]. Again, he achieved 100% chimerism following transplantation[242], and his AML went into remission[242, 253]. Following his first transplantation, Mr. Brown was never put on ART again and has not experienced viral rebound to this day[254]. Extensive sampling of blood, rectal tissue, gut tissue, cerebral spinal fluid (CSF), lymph node, and brain tissue was performed and analyzed by multiple labs for detection of HIV-1[253, 255]. These analyses revealed no replication-competent virus, no HIV-1 RNA, no HIV-1 DNA, no cells expressing CCR5, and a waning HIV-1-specific immune response[255]. These findings have collectively led to Timothy Ray Brown being labeled as the first and only person to be cured of HIV-1.

The question still remains: how did this course of treatment and ASCT cure this one individual of HIV-1? Three main hypotheses have been proposed as to how Mr. Brown was cured of HIV-1 and has not experienced viral rebound following ASCT. His clinical success could be due to: (i) the successful ASCT from a CCR5delta32 homozygous donor, (ii) the intensive pre- and post-therapy conditioning, involving myeloablative TBI, multiple rounds of chemotherapy, and immunosuppression, or (iii) transplant-associated toxicities and complications, such as GVHD and leukoencephalopathy, which led to the further destruction of remaining recipient cells[253, 256]. Attempts have been made to recapitulate the successful elimination of HIV-1 from Mr. Brown. Some of those trials are explained in the next section.

### 1.3.3 The Boston Patients and other human and non-human primate stem cell transplant attempts to cure HIV-1

Stem cell transplantation is not an approved treatment for those living with HIV-1 in the era of ART due to the significantly high mortality rates associated with these procedures[257, 258]. However, in HIV-1-infected individuals with underlying malignancies, stem cell transplantations are sometimes required as a last resort to eliminate the cancer. In 2013, Henrich et al. reported on two HIV-1-infected individuals with underlying lymphomas who underwent ASCT to determine the impact this procedure had on HIV-1 reservoirs and persistence[259]. Because these studies were conducted in Boston, Massachusetts, these individuals became known as the "Boston Patients." Similar to Timothy Ray Brown, both of these individuals were homozygous for the CCR5delta32 mutation and developed their cancer while on suppressive ART.

The first patient relapsed after standard chemotherapy and again after salvage chemotherapy and autologous HSCT. This indicated the need for an ASCT. The patient underwent RIC and received a single human leukocyte antigen (HLA) mismatched HSCT from an unrelated donor. He also received post-transplantation anti-GVHD medication. The second patient underwent standard chemotherapy and then received a new diagnosis of stage IV mixed-cellularity Hodgkin disease. He underwent unsuccessful chemotherapy, which then led to an autologous HSCT. Unfortunately, this patient suffered many complications, including persistent thrombocytopenia and anemia. Ultimately, he was diagnosed with myelodysplastic syndrome with multilineage dysplasia, which is a blood disorder in which bone marrow cells are unable to develop or develop properly[260]. This condition necessitated an ASCT, which he received from a matched sibling donor after RIC. This donor also received post-transplantation anti-GVHD medication. Both patients achieved full chimerism after seven months.

Following transplantation, both of the Boston Patients developed clinically significant GVHD requiring immunosuppressive treatments. Unlike Mr. Brown, both patients underwent RIC conditioning instead of myeloablative TBI. Furthermore, these individuals remained on ART after ASCT. Both patients had detectable HIV-1 DNA in PBMCs pre- and shortly post-transplantation, as well as low level detectable HIV-1 RNA in plasma shortly after transplantation. However, both HIV-1 DNA and RNA levels became undetectable at later time points.

To understand the full effect on HIV-1 persistence that ASCT had in these two patients, 4.3 years (patient 1) and 2.6 years (patient 2) post-transplantation, extensive blood and tissue sampling was performed, followed by an analytic treatment interruption (ATI)[261]. Neither patient had detectable HIV-1 DNA or RNA. Both patients also had no significant HIV-1-specific cellular immune responses. Furthermore, microchimerism analyses revealed that less than 0.0010% of PBMCs were of host origin. Both patients were closely monitored following the ATI. Patient 1 had detectable plasma HIV-1 RNA 84 days after ATI and patient 2 had high levels of plasma HIV-1 RNA after developing an illness. ART was immediately re-administered to both individuals, who quickly achieved full suppression again.

These findings were quite devastating to the HIV field for many reasons. Most importantly, it revealed that despite having a $\geq$ 3-$\log_{10}$ reduction in the circulating proviral reservoir, as determined by the microchimerism data and undetectable HIV-1 DNA in tissue samples by ultrasensitive methods, viral rebound still occurred. While most individuals who undergo a treatment interruption without ASCT experience viral rebound within 1-8 weeks[190-195], these two patients experienced a delayed rebound at 12 and 32 weeks[261]. Following these results, Hill et al. predicted with mathematical modeling and available clinical data that an

approximately 2000-fold reduction in the latent reservoir is required to achieve remission without ART for 1 year[262]. They also suggest that a greater than 10,000-fold reduction is likely required to prevent viral rebound altogether (i.e. to achieve a functional cure). Additional clinical studies with scheduled treatment interruptions will be needed, however, to determine if these predictions are correct[263].

Around the same time that the Boston Patient study was being conducted, Dr. Guido Silvestri's group evaluated the affect of the conditioning regimen on clinical outcome and viral persistence following ASCT in a rhesus macaque model using a chimeric simian immunodeficiency virus (SIV) that contained HIV-1 RT (RT-SHIV)[256]. In this study, three RT-SHIV-infected, ART-suppressed animals underwent autologous HSCT using cells that were collected prior to infection. These study animals underwent myeloablative TBI prior to transplantation, similarly to that given to Mr. Brown. These animals did not, however, receive antithymocyte globulin or chemotherapy[242]. These animals were compared to three control animals on the same ART regimen but that did not receive conditioning or autologous HSCT. Importantly, all six animals had equally suppressed viral loads to below the limit of detection. After successful engraftment had occurred (40-75 days post-transplantation), all six animals underwent an ATI. Along with the control animals, two of the three transplanted animals experienced rapid rebound of virus, as early as one week post-treatment interruption[256]. The third transplanted animal did not experience viral rebound within 14 days, when it had to be euthanized for clinical reasons. SIV DNA was detected in all three transplanted animals at similar levels to those seen in the control animals in several tissues following necropsy. This study revealed that conditioning alone is insufficient to prevent viral rebound following treatment interruption.

Given these findings, it seems clear that both the use of a CCR5delta32 homozygous donor, along with the associated toxicities and complications from the conditioning and ASCT, were significant contributors to the clearance of HIV-1 in Mr. Brown. Additional studies have been conducted to evaluate the role of using a CCR5delta32 homozygous donor to eliminate HIV-1 infection. At least six additional individuals have undergone ASCT from a CCR5delta32 homozygous donor[254, 264, 265]. Unfortunately, all six patients died within 1 year of transplantation from recurrent malignancy, GVHD, or infection. One individual also experienced rebound of X4-tropic virus, providing a proof-of-concept that transplantation with CCR5-defective cells could result in a switch to or outgrowth of X4-tropic variants. These findings demonstrate the unrealistic feasibility of using CCR5delta32 homozygous donors and ASCT to eliminate HIV-1. Mr. Brown is a truly unique case of HIV-1 cure.

Other approaches to transplant CCR5-deficient cells into HIV-1-infected individuals are currently being pursued. One approach is to use umbilical cord blood stem cells instead of peripheral blood or bone marrow stem cells[266]. While standard stem cell transplantations require closely matched HLA types to prevent graft rejection[267, 268], complete HLA matching is not required when using umbilical cord blood stem cells[266, 267]. Given the rare frequency of individuals who are homozygous for the CCR5delta32 gene in addition to the need for HLA matching in stem cell transplantation, the odds of finding matched donors for HIV-1 individuals are very low. To overcome this limitation, a collaborative effort has been made to generate a cord blood inventory from CCR5delta32 homozygous donors to be used at later times for HSCT. Depending on the cell dose required for transplantation, the projected probability of finding a match from this inventory for a Caucasian patient ranges from 28% - 86%, which is significantly higher than through alternative approaches.

Another alternative approach is to genetically modify hematopoietic stem cells to render them resistant to HIV-1 infection. Several methods to genetically modify CCR5 or the expression of CCR5 have been assessed in pre-clinical studies using targeted ribozymes, RNA decoys, transdominant mutants, RNA interference (RNAi) through the use of small interfering RNAs (siRNAs), anti-sense RNAs, microRNAs, or short hairpin RNAs (shRNAs), zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENS), and engineered clustered regularly interspersed palindromic repeats (CRISPR) coupled to a CRISPR-associated (Cas) nucleases[269-300]. Many of these studies were performed in humanized mouse models and demonstrated that moderate to high levels of resistance to HIV-1 infection with an R5-tropic virus could be achieved following infusion or transplantation of CCR5-deficient cells[269-280, 296, 297, 299]. Many of these approaches have also been evaluated in HIV-1-infected individuals[300-305]. In these studies, it was generally found that infusion or transplantation of CCR5-deficient cells containing an anti-HIV-1 vector was safe and well tolerated. There have been limitations to these studies, however, largely due to low transduction efficiency and insufficient sustainability of the modified cells. Improved techniques to overcome these challenges are under development.

Clinical trials evaluating many of these approaches to eliminate HIV-1 are in progress[282, 306]. Despite some success, and little to no reporting of major adverse events related to treatment, there is still significant concern that gene therapy approaches will lead to off-target effects and oncogenesis[307]. Furthermore, despite the decreased risk of using RIC regimens versus fully myeloablative therapy, there is still a risk treatment-related toxicities, which outweighs that of current ART regimens[151, 231, 308]. The future of these therapeutic strategies as an effective and acceptable approach to eradicate HIV-1 is still unknown.

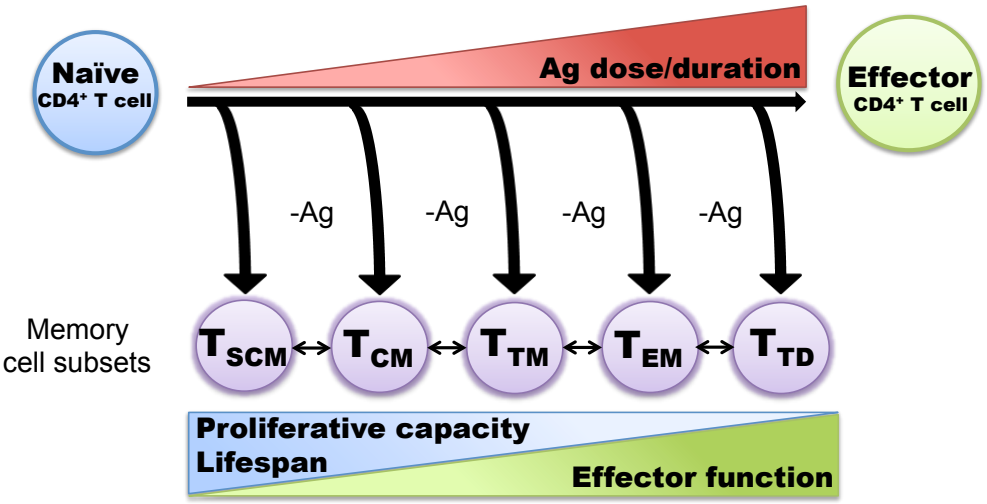
## 1.4 HIV-1 LATENCY

### 1.4.1 CD4+ T cell subsets and the composition of the HIV-1 latent reservoir

The recovery of replication-competent HIV-1 from individuals on suppressive ART led to the discovery of HIV-1 persistence in a latent reservoir of rCD4 T cells[155-157]. Shortly thereafter, it became clear that the majority of HIV-1 DNA-containing cells were resting memory CD4+ T cells, while HIV-1 DNA was found at a much lower frequency in $T_N$ cells[309-316]. In 2009, a detailed analysis of HIV-1 DNA in different CD4+ memory T cell subsets, in addition to $T_N$ cells, was published, demonstrating that all rCD4 T cell subsets contribute to the pool of latently infected cells in patients on long-term ART, albeit to varying degrees[317]. At the time of this publication, there were 4 known distinct memory T cell subsets, termed central memory ($T_{CM}$), transitional memory ($T_{TM}$), effector memory ($T_{EM}$), and terminally differentiated ($T_{TD}$) cells. These memory cell subsets are distinct from each other, as well as from $T_N$ cells, based on phenotype, function, and location throughout the body. Ex vivo distinction between the $T_N$ and memory T cell subsets largely relies on characterization of several cell surface markers, including CD45RO, CD45RA, CCR7, CD62L, and CD27 (Fig. 3).

CD45, also known as common leukocyte antigen[318], is a tyrosine phosphatase[319, 320] important in T cell signaling and development[321]. Multiple CD45 isoforms exists as a result of alternative RNA splicing[322]. In humans, it has been shown that the antigen-inexperienced $T_N$ cells express the high molecular weight CD45RA isoform; however, upon T cell activation, expression of CD45RA is down-regulated and the low molecular weight CD45RO isoform becomes expressed[321, 323-326]. Expression of CD45RO is a marker of both memory and effector cells[321]. Because effector cells express T cell activation markers, such as CD25, CD69, or HLA-

DR, they are easily distinguishable from the memory cell population. Therefore, $T_N$ and memory cell populations can be separated based on a lack of T cell activation and variable expression of CD45RA and CD45RO, respectively.




| | | | | | | |
|---|---|---|---|---|---|---|
| CD45RO | - | - | + | + | + | - |
| CD45RA | + | + | - | - | - | + |
| CCR7 | + | + | + | - | - | - |
| CD27 | + | + | + | + | - | - |
| CD95 | - | + | + | + | + | + |
| CD62L | + | + | + | - | - | - |

**Figure 3: CD4+ T cell maturation and generation of memory T cell subsets.** Ag = antigen. –Ag = removal of antigen.

CD62L (also known as L-selectin) and CCR7 are both essential for lymphocyte migration to secondary lymphoid organs[327]. CD62L is a selectin family adhesion molecule expressed on circulating lymphocytes[328]. CD62L is important for lymphocyte tethering to the vascular networks that surround secondary lymphoid organs, such as the lymph nodes[328-330]. Following lymphocyte tethering mediated between CD62L and its ligands, collectively known as peripheral-node addressin (PNAd) adhesion molecules[329], the interaction between CCR7 with its ligands, either CCL19 or CCL21, leads to integrin activation, cell adhesion, and transmigration into the lymph node[331, 332].

In 1999, it was shown that either expression of or lack of expression of CCR7 could distinguish memory cells into two distinct groups[333]. CCR7+ cells express lymph node homing receptors and lack immediate effector functions, while the CCR7- cells express receptors for migration into tissues and are capable of immediate effector function. These two memory cell subsets became known as the $T_{CM}$ and the $T_{EM}$ cells, respectively. Further characterization of the expression of CCR7 in combination with CD62L in mice infected with lymphocytic choriomeningitis virus revealed an intermediate or $T_{TM}$ subset that lacked expression of CD62L but maintained expression of CCR7[334].

CD27 is a member of the tumor necrosis factor (TNF) receptor superfamily[335], that was known to distinguish highly differentiated versus minimally differentiated memory T cells[336]. Interaction between CD27 and its ligand, CD70, plays an important role in T cell survival and proliferation[337]. Additional analysis of CD27, in conjunction CD62L and CCR7, led to further delineation of the memory T cell subsets, with the additional identification of $T_{TD}$ memory cells[338].

In 2011, a new memory T cell subset was identified in humans, termed the stem cell-like memory cells ($T_{SCM}$)[339]. $T_{SCM}$ cells fall between $T_N$ and $T_{CM}$ cells, both phenotypically and functionally[339]. These cells maintain expression of CD45RA, CD62L, CCR7, and CD27, while lacking expression of CD45RO. These cells were also found to be antigen-experienced and expressed high levels of CD95, IL-2Rβ (CD122), CXCR3, and LFA-1, which are all characteristic of memory cells[339]. Under conventional subset isolation or flow gating strategies, $T_{SCM}$ cells falls within the $T_N$ cell population. Therefore, the additional characterization of CD95 and/or CD122 is required to distinguish $T_{SCM}$ cells from $T_N$ cells. $T_{SCM}$ cells are an infrequent cell type, contributing to < 5% of the $T_N$ cell population in the peripheral blood[340-345].

In addition to surface phenotype, the $T_N$ and memory T cell subsets are distinguished by many physiological features (Fig. 3). By definition, $T_N$ cells are antigen-inexperienced and require antigen, co-stimulation from an antigen presenting cell, and signals from the cellular microenvironment to become activated[346, 347]. These cells lack effector functions and circulate through secondary lymphoid tissues, via the blood, allowing for efficient immune surveillance of infection[346]. Among the rCD4 T cell subsets, $T_N$ cells are the least differentiated but have the highest proliferative capacity[338, 348]. These cells are also able to generate into effector cells as well as any of the memory T cell subsets, depending on the dose and duration of antigenic stimulation[348, 349].

The memory T cell subsets are distinguished by proliferative capacity, effector function, telomere length (as a marker for previous cell divisions), cytokine secretion, chemokine receptor expression, and many other physiological distinctions. The $T_{SCM}$ cells were found to have an enhanced capacity for self-renewal and the highest proliferative capacity among all of the memory T cell subsets[339]. These cells were also characterized as the least differentiated of the memory cells and, similar to $T_N$ cells, are able to maturate into the other memory cell types, as well as effector cells, upon stimulation[339]. The $T_{CM}$ cells are the second least differentiated memory T cell subset. They have a lower proliferative capacity than the $T_{SCM}$ cells but have enhanced immune recall upon secondary stimulation and a greater number of effector functions[339]. The $T_N$, $T_{SCM}$, and $T_{CM}$ cells express similar secondary lymphoid homing receptors that allow them to circulate through the blood and lymphoid tissues. The $T_{TM}$ cells are more differentiated than the $T_{CM}$ cells but less differentiated than the $T_{EM}$ cells. They do not express the lymph node homing receptor CD62L, but have increased expression of tissue homing receptors such as CCR6 and CXCR3[348, 350]. These cells have a reduced proliferative capacity in

comparison to $T_{CM}$ cells as well as contain shorter telomeres, but have a quicker immune response and greater effector functions[338, 350]. $T_{EM}$ cells lack expression of CD62L, CCR7, and CD27 and are largely located in peripheral tissues. These cells have a diminished ability to proliferate in comparison to the other memory cell subsets, but have the shortest secondary response time to antigen and have the highest functional capacity[350]. $T_{TD}$ cells are an end-stage phenotype with greatly reduced telomerase activity, almost entirely lacking the ability to proliferate[338]. These cells lack all lymphoid homing receptors and thus make up only a small fraction (1-3 %) of circulating CD4+ T lymphocytes[317, 338, 351]. The $T_{TD}$ cells will not be a focus of discussion in this thesis.

All of these different rCD4 T cell subsets have been implicated in HIV-1 infection. While all of these cells express CD4, the HIV-1 co-receptors, CCR5 and CXCR4, are variably regulated on these different cell subsets, which likely contributes to their susceptibility to HIV-1 infection. There is a maturational relationship between both CCR5 and CXCR4 expression among the different T cell subsets. CXCR4 is most highly expressed on $T_N$ cells, decreasing in expressing to the $T_{EM}$ cells ($T_N > T_{SCM} > T_{CM} > T_{TM} > T_{EM}$), whereas expression of CCR5 is the opposite, being almost completely absent on $T_N$ cells to being expressed on the majority of $T_{EM}$ cells[343, 344, 352].

While essentially all studies to date that have evaluated the frequency of HIV-1 DNA in different CD4+ T cell subsets have found that $T_{CM}$ and/or $T_{TM}$ cells contain the highest levels of HIV-1 DNA, variations in the distribution of HIV-1 DNA among the different CD4+ T cell subsets have been associated with different rates of disease progression and clinical benefit. During HIV-1 infection, a fraction of individuals are able to naturally suppress viral replication or do not progress to AIDS in the absence of ART. These individuals are termed elite controllers

(EC), viremic non-progressors (VNP), and long-term non-progressors (LTNP) (broadly termed HIV-1 controllers here)[353]. While there are many different clinical features that distinguish ECs, VNPs, and LTNPs from each other and from normal or rapid progressors, including viral load, CD4+ T cell count, and duration of infection, how these three groups of HIV-1-infected individuals are able to control infection and prevent or delay disease progression is not well understood. Decreased infection in $T_{CM}$ cells has been observed in ECs[354], as well as LTNPs who express the protective HLA-B27/B57 alleles[355]. Furthermore, limited infection in both $T_{SCM}$ and $T_{CM}$ cells has been found to correlate with a lack of disease progression in VNPs[345]. Although differences in HIV-1 infection of $T_N$ cells between HIV-1 controllers and progressors has not been directly observed, one major finding is that while lymphopoiesis and thymic output becomes severely impaired in progressive HIV-1 infection[66, 356, 357], these functions are largely preserved in HIV-1 controllers[353, 358, 359]. This results in a stable level of $T_N$ cells and enhanced immune function[353].

Initiation of ART during acute HIV-1 infection has been shown to reduce the size of the latent reservoir[360-372]. Two studies have shown that some individuals who initiate ART during primary HIV-1 infection are able to control infection following treatment interruption for an extended period of time[360, 362]. In 2013, the ANRS VISCONTI Study Group reported that in some patients who received ART within 10 weeks of primary HIV-1 infection and remained on ART for a median of 3 years, viremia could be controlled for at least 24 months following treatment interruption[360]. These individuals were termed post-treatment controllers. Interestingly, HIV-1 DNA could only be detected in $T_N$ cells from 2 of 11 individuals, which suggests that the limited HIV-1 reservoir in $T_N$ cells may be important for long-term control of viral replication.

Furthermore, in these patients the short-lived $T_{TM}$ cells, and not the long-lived $T_{CM}$ cells, appeared to be the major contributor to the latent reservoir[360].

In 2015, the ANRS OPTIPRIM Study Group compared the effects of ART initiated during primary HIV-1 infection when continued for 2 or 6 years with the post-treatment controllers identified in the VISCONTI study[362]. This study found similarly low levels of HIV-1 infection of $T_N$ cells (HIV-1 DNA was detected in 4 of 9 patients) as well as $T_{CM}$ cells, particularly in the 6-year treatment group. Two individuals who displayed similar characteristics as the post-treatment controllers underwent a treatment interruption. One individual controlled viral replication to < 20 copies/mL plasma HIV-1 RNA up to the 24 month follow-up, while the second individual was able to partially control HIV-1 replication to 530 copies/mL plasma HIV-1 RNA up to 18 months following treatment interruption compared to > 100,000 copies/mL and > 1,500 copies/mL, respectively, prior to treatment initiation. These findings were in accordance with the VISCONTI study, demonstrating that early initiation of ART could limit the size of the latent HIV-1 reservoir, particularly in $T_N$ and $T_{CM}$ cells, and lead to post-treatment control following prolonged ART prior to treatment interruption. These studies collectively highlight the importance of long-lived memory cells in the long-term control and maintenance of HIV-1 infection.

### 1.4.2 HIV-1 latency is maintained at the level of transcription

Two general forms of HIV-1 latency have been described in HIV-1-infected individuals, based on whether or not the virus has integrated into the host genome. Pre-integration latency results from the inability of the viral genome to integrate into the host cell genome[373]. This can occur from incomplete reverse transcription, impaired nuclear import, or incomplete integration,

resulting in the formation of 1-LTR circles, 2-LTR circles, or linear unintegrated HIV-1 DNA[374, 375]. These unintegrated forms of HIV-1 DNA are labile and do not persist, indicating that pre-integration latency does not contribute to the long-term latent reservoir in HIV-1-infected individuals on ART[376]. Post-integration latency results from viral DNA integration into the host cell genome whereby viral transcription is then blocked[377]. Unlike pre-integration latency, post-integration latency is highly stable and can persist for the life of the infected cell. While incompletely understood, many host factors, pathways, and mechanisms that contribute to HIV-1 latency have been resolved, largely through the use of cell line models or primary cell models of HIV-1 latency, which will be discussed more extensively below.

The HIV-1 5' LTR contains several DNA binding sites for cellular transcription factors such as Sp1, activator protein-1 (AP-1), NF-κB, and nuclear factor of activated T cells (NFAT). The chromatin organization around the HIV-1 promoter plays a key role in regulating viral gene transcription. Irrespective of where HIV-1 integrates into the host genome, nucleosomes, in particular nuc-0 and nuc-1, are bound to the HIV-1 5'LTR at fixed positions[378]. Nuc-1 prevents transcriptional elongation as it is positioned just downstream of the transcription start site[379]. In latently infected cells, the nucleosomes on the HIV-1 5' LTR are typically found to contain deacetylated and both di- and trimethylated histones (H3K9 and H3K27), all of which are associated with repressive heterochromatic features[380, 381]. Both histone deacetylases and histone methyltransferases themselves have also been found to be associated with the proviral promoter in latently infected cells[380-387].

In addition to the positioning of nuc-1, transcriptional elongation is also inhibited by the presence of negative transcription elongation factor (NELF) and DRB-sensitivity inducing factor (DSIF), which induce RNA pol II pausing, resulting in abortive transcription[388]. In rCD4 T cells

or metabolically inactive cells in general, RNA pol II contains an unphosphorylated C-terminal domain (CTD), which allows RNA pol II to initiate transcription but lacks elongation abilities[389]. In order for processive transcription to occur, the CTD of RNA pol II becomes hyperphosphorylated, largely through the kinase subunit of the positive transcription elongation factor b (P-TEFb). P-TEFb is a heterodimeric protein composed of cyclin-dependent kinase 9 (CDK9) and its regulatory partner cyclin T1. In rCD4 T cells, cyclin T1 is present at low levels and, while CDK9 is constitutively expressed, it is found in an inactive form[390-393]. Additionally, both P-TEFb subunits are sequestered in a 7SK small nuclear ribonucleoprotein complex (7SK snRNP), preventing activation of RNA pol II or other regulatory proteins. In addition to phosphorylating the CTD of RNA pol II, P-TEFb also phosphorylates NELF and DSIF, causing dissociation from the HIV-1 promoter and relieving transcriptional repression.

Efficient HIV-1 transcription requires not only association of the HIV-1 Tat protein with the TAR element, but also association with P-TEFb[394]. P-TEFb can be released from the 7SK snRNP either through recruitment by Tat or by the chromatin-binding bromodomain protein BRD4[395-397]. Thus, BRD4 acts as a competitive inhibitor of Tat for binding to P-TEFb. Therefore, during latent HIV-1 infection, when P-TEFb levels are low and BRD4 levels are high, HIV-1 transcription is blocked.

In contrast to activated CD4+ T cells, in which transcription factors are highly expressed in the nucleus, many transcription factors, including those important for HIV-1 transcription (NF-κB, NFAT, and AP-1) are sequestered in the cytoplasm in an inactive form in rCD4 T cells[398, 399]. NF-κB dimers are sequestered through interaction with inhibitory IκB proteins. NFAT is maintained in the cytoplasm in a phosphorylated, inactive state, unable to enter the nucleus. AP-1 is a heterodimeric protein composed of c-Fos, c-Jun, or ATF. The subunits that

make up AP-1 are functionally inactive in rCD4 T cells and cannot form active heterodimers.

While there are likely many additional factors that contribute to the control of HIV-1 latency that either have not been discussed above or have not yet been identified, the overall physiological environment of a rCD4 T cell provides a perfect setting for HIV-1 to maintain a lifelong persistent infection.

### 1.4.3 Methods to study HIV-1 latency

#### 1.4.3.1 Cell line models

Cell line models are an attractive and cost-effective way to study HIV-1 latency because they are generated from immortalized cell lines derived from the expansion of cells containing a single integration event, allowing for large scale, reproducible studies. Several different cell line models of HIV-1 latency have been developed to serve different experimental purposes. Some cell lines harbor full-length HIV-1 while others contain only minimal viral components. One of the greatest benefits to using a cell line model is that many of the integrated viral genomes also contain a reporter gene (such as luciferase or GFP) that is expressed only when viral transcription becomes activated, allowing for quick and easy analysis of latency reversal. As such, the use of cell line models has made large-scale screenings and mechanistic studies of latency reversing agents (LRAs) possible.

The initial observation that HIV-1 could form a latent infection came in 1986 from in vitro infection experiments of the transformed T cell line, A3.01[400]. It was shown that upon infection of A3.01 cells with HIV-$1_{LAV}$, over 95% of these cells became productively infected and the majority of them died[400]. However, a small fraction of the infected cells survived, and over long-term culture, these surviving cells were no longer producing virus or synthesizing viral

proteins[400]. Importantly, the authors demonstrated that these cells were still capable of producing virus following stimulation for up to 90 days, indicating that these cells contained an HIV-1 provirus that was in an inactive, latent state.

The first four cell lines of HIV-1 latency to be generated were U1 cells[401], a promonocytic cell line; ACH-2 cells[402], a T cell line; J1.1 cells[403], also a T cell line; and OM-10.1 cells[404], a promyelocyte cell line. All four cell lines contained a full-length HIV-1 clone and were characterized by having little to no expression of HIV-1, but could be induced to produce virus when stimulated with TNF-α[402-404] or mitogens such as phorbol-12-myristate 13-acetate (PMA)[401]. Early studies using these cell lines provided a great deal of insight into the control and regulation of HIV-1 latency.

Although these cell lines have yielded tremendous advances in the field of HIV-1 latency research, they are not without their flaws. It was later identified that the latency phenotype in U1 and ACH-2 cells resulted from mutations in Tat[405] or in the TAR RNA stem loop[406], respectively, which fail to recapitulate the complexity and natural state of HIV-1 latency in vivo. J1.1 cells express reduced surface levels of CD3 and contain defects in both IL-2 production and calcium mobilization following stimulation, rendering them defective in T cell receptor signaling[403]. Furthermore, it has recently been shown that these three cell types contain multiple viral integration sites, indicating that these cells are not clonal and have undergone some level of viral replication over time[407].

More recently, the J-Lat cell lines were developed, which are a series of T cell lines of HIV-1 latency[408, 409]. The original J-Lat cell lines were generated using a minimal, non-replication competent HIV-1 vector that lacked several genes necessary for in vivo replication[409]. Additional J-Lat cell lines were created by infecting Jurkat cells with a full-length HIV-1 vector

containing a frameshift mutation in *env* and insertion of the enhanced green fluorescent protein (EGFP) gene in place of *nef*[408]. Through the generation of the J-Lat cell clones, several were identified that did not produce virus (measured by p24 enzyme-linked immunosorbent assay [ELISA]), did not produce viral proteins (measured by western blot analysis), and were GFP negative, indicating that these cells were transcriptionally silent and in a state of post-integration latency[408]. These features made these cell lines a more convenient way to study HIV-1 latency through the addition of the GFP reporter gene.

Numerous other cell line models of HIV-1 latency have been developed, each containing unique integration sites, and were generated using different viral strains and viral constructs to serve different research purposes. Additional cell line models of HIV-1 latency will not be discussed further here.

#### 1.4.3.2 Animal models

Animal models used to study HIV-1 latency and persistence offer unique and invaluable features that cannot be achieved by any other methods. The best and most widely used animal models to study HIV-1 latency are the non-human primate (NHP) models[410]. Many NHP models of HIV-1 infection have been developed to serve different purposes, including the use of different NHPs, such as rhesus macaques, pigtailed macaques, cynomolgus macaques, African green monkeys, and sooty mangabeys[410]. Many species of African monkeys and apes are natural hosts for SIV and therefore do not develop an AIDS-like illness[411]. Asian macaques, however, are not natural hosts for SIV infection and tend to develop simian AIDS, displaying high plasma viremia, a loss of CD4+ T cells, and opportunistic infections before death[412]. The NHP models of HIV-1 infection range from spontaneous control to rapid disease progression[413-415]. This wide spectrum of clinical manifestations is dependent on the NHP model, as well as the virus being used[410].

These features allow for a vast diversity of studies that can be conducted to recapitulate human HIV-1 infection.

SIV infection of Asian macaques shares many key features of HIV-1 infection in humans that make this model a great tool for latency research (reviewed in [410]). Following administration of ART, plasma HIV-1 RNA can become suppressed to below the limit of detection. Furthermore, SIV DNA integration sites[416-418] and cellular distribution between the blood, lymph nodes, and mucosal sites[419-421] are similar to that seen with HIV-1 infection. These characteristics suggest that there are similar reservoir dynamics between HIV-1 infection in humans and SIV infection in macaques making this model particularly useful in latency research.

The greatest benefits to using the NHP models to study HIV-1 latency and persistence are the controlled conditions in which the experiments are conducted. Drug regimens and dosing can be carefully administered and monitored to ensure adherence. With NHPs, it is not only possible to control the virus being used for infection, but different modes of transmission can also be evaluated. Given the relatively large size of NHPs, frequent tissue and blood samples can be collected to monitor disease and viral dissemination. To this end, macaque models of HIV-1 latency have greatly advanced the field of HIV-1 latency research, providing a greater insight into viral dissemination following primary infection, establishment of the latent viral reservoir, and in vivo evaluation of novel LRAs.

One major question that had remained unanswered was how quickly the latent viral reservoir is established following HIV-1 infection. Rhesus macaques were infected intrarectally with SIV and subsequently treated with daily ART 3, 7, 10, or 14 days following infection[370]. It was shown that although the animals treated 3 days post-infection never achieved measurable plasma HIV-1 RNA levels, virus in the animals rebounded following treatment interruption and

HIV-1 DNA had disseminated in lymph nodes and gut-associated lymphoid tissue (GALT) but not in PBMCs. This demonstrated that the HIV-1 latent reservoir is established within days of infection, much earlier than previously thought. These findings helped explain the ultimate results of the once-thought cured 'Mississippi baby'[422]. The so-called 'Mississippi baby' was a perinatally infected child who received ART 30 hours after birth and remained on ART through 18 months of age. ART was discontinued between 18 – 46 months of age but the child did not have detectable plasma HIV-1 RNA measured by ultrasensitive assays. It was unclear, however, if latent viral reservoirs had been established prior to initiation of ART just 30 hours after birth. At 46.4 months of age, plasma viremia rebounded, demonstrating that despite very early initiation of ART, a latent reservoir had been established, and although significantly delayed, ultimately replicating virus reemerged in the absence of therapy[423].

Another main question largely not addressed in humans is whether or not low-level viral replication occurs in tissues during successful ART and contributes to persistent plasma viremia that is seen in HIV-1-infected individuals. One study published in 2015, through the use an RT-SHIV macaque model, clearly demonstrated that ongoing viral replication does not occur in tissues of ART-suppressed animals[173]. In this study, single-genome sequencing was performed on HIV-1 RNA and DNA from 11 anatomical sites obtained from 4 RT-SHIV-infected pigtail macaques. The authors found no differences in viral populations between the plasma and tissues as well as no sequence divergence in either the plasma or between the tissues of the two ART-treated animals. This extensive sampling and sequencing analysis definitively demonstrated a lack of viral evolution while on ART, suggesting that viral replication was not taking place.

Studies such as these provide a great deal of insight into the in vivo dynamics of HIV-1 latency and the latent reservoir that cannot be obtained in humans. While studies in NHPs are

limited due to both cost and space required to maintain the animals, the continued use of NHPs in HIV-1 latency and reservoir research will surely provide invaluable information that cannot be obtained by other means.

In addition to the NHP models, there are also several humanized mouse models of HIV-1 that have been used for latency studies. Humanized mouse models represent an attractive alternative to NHP models due to the significantly reduced cost of purchasing, housing, and maintaining the animals. They are also much smaller and easier to work with than NHPs. In general, humanized mice are generated by reconstituting immunodeficient mice with human cells with or without human fetal tissues to allow them to develop a human immune system[410, 424]. It has been shown in several different humanized mouse models that HIV-1 latency can be established at a similar or greater frequency than that seen in humans[425-429]. In the context of HIV-1 latency and the latent viral reservoir, humanized mouse models have been widely used to study in vivo evaluation of LRAs[430-436], and gene therapy approaches to reduce the latent reservoir or block HIV-1 infection using ZFNs[273, 274, 279, 437], siRNAs[269, 270, 296-299], shRNAs[272, 276, 277, 280, 438], or ribozymes[275].

There are several limitations to using humanized mouse models, however that may limit their use for HIV-1 latency research. The HIV-1 latent reservoir is highly complex, involving many different CD4+ T cell subsets, which are distributed throughout the body of an HIV-1-infected individual on ART. In humanized mouse models, the precise composition and distribution of human immune cells has not been fully evaluated. It has been shown, however, that there is incomplete colonization of mouse tissues with human cells, and that there are incomplete B and T cell responses, which may be important for the control of viral replication[424, 439]. Development of certain humanized mouse strains also require the use of human fetal tissue

and requires surgical procedures to implant the fetal tissue[410, 424]. Given the current limitations to humanized mouse models for HIV-1 latency research, improved humanized mouse models with enhanced human immune cell distribution and function are currently being pursued.

#### 1.4.3.3 Primary cell models

Tremendous advances have been made in the area of HIV-1 latency research through the use of primary cell models. The frequency of latently infected cells is very low, approximately 100 copies of HIV-1 DNA per $10^6$ rCD4 T cells measured in asymptomatic patients or patients on ART, which makes this population difficult to study[158, 440, 441]. To further complicate the study of these rare cells ex vivo, there are no known phenotypic markers that would allow for the enrichment of latently infected cells. To circumvent the rarity of latently infected cells in vivo, a large effort has been made to develop primary cell models of HIV-1 latency in which higher levels of infection can be achieved. Several models have been developed to date with the primary goal of studying LRAs and mechanisms of both the establishment and reversal of HIV-1 latency[442-451]. Unlike cell line models or NHP models used to study latency and persistence, primary cell models allow for the investigation of HIV-1 latency in human, clinically relevant cell types. All of these models can loosely be divided into two groups: one group which relies on infection of activated CD4+ T cells prior to the establishment of HIV-1 latency, and one which relies on direct infection of rCD4 T cells.

The first primary cell models of HIV-1 latency that were developed utilized methods of direct infection of rCD4 T cells in order to maintain phenotypic and biologic integrity. There are many limitations, however, to direct infection of rCD4 T cells, due to host restriction factors and blocks imposed by the cellular environment that make these cells non-permissive to infection (reviewed in [452, 453]). The actin cytoskeleton is known to be a key regulator in many early HIV-1

processes including viral entry, reverse transcription, intracellular trafficking, and integration[454-456]. In rCD4 T cells, cortical actin is static and is restrictive to HIV-1 infection due to actin and its regulators being in an inactive state[457]. It has been shown that binding of HIV-1 gp120 to CXCR4 is able to enhance early stages of infection in rCD4 T cells by activating cofilin, which also results in actin polymerization and enhanced actin dynamics[454, 455, 458]. In rCD4 T cells, static cortical actin poses a post-entry restriction to HIV-1 infection, primarily in the case of R5-tropic viruses.

Another restriction is imposed by the sterile alpha motif and histidine/aspartic acid domain-containing protein 1 (SAMHD1), a deoxynucleoside triphosphate triphosphohydrolase (dNTPase), which is highly expressed in an activated form (unphosphorylated) in rCD4 T cells[459]. This protein was originally identified in myeloid cells as an HIV-1 restriction factor that blocked HIV-1 reverse transcription by depleting cellular dNTP pools[460-463]. More recently, it has been suggested that SAMHD1 inhibits HIV-1 infection by degrading the viral genomic RNA through the RNase activity of SAMHD1[464, 465]. However, this second mechanism has not been shown to contribute to the antiviral activity of SAMHD1 in cells[466]. Treatment of rCD4 T cells with the homeostatic cytokines IL-2 or IL-7 have been shown to inactivate SAMDH1 by inducing phosphorylation at threonine 592, making these cells more permissive to HIV-1 infection[467]. It has also been shown that treatment of rCD4 T cells with either IL-2 or IL-7 enhances HIV-1 infection[468], possibly through alleviation of the SAMHD1 restriction.

The first primary cell model of HIV-1 latency was published in 2005 by Una O'Doherty's group[449]. In this study, the authors used spinoculation to infect primary rCD4 T cells. Spinoculation is centrifugal inoculation, in which cells and virus are centrifuged for 2 hours in a small volume to ensure adequate virion-cell interaction. Using this method, they found

that a small fraction of cells became latently infected, as measured by integrated HIV-1 DNA, but they lacked intracellular Gag expression. They noted that both reverse transcription and integration were delayed when compared to infection of activated CD4+ T cells. When stimulated with either IL-7 or anti-CD3/CD28, a fraction of the latently infected cells produced intracellular Gag, suggesting a reversal of latent proviruses. This study demonstrated that HIV-1 latency could be established in vitro by direct infection of rCD4 T cells without T cell activation. A subsequent paper was published demonstrating that spinoculation increases actin dynamics and cofilin activity, likely due to centrifugal stress[469]. This finding warrants caution when interpreting results using spinoculation techniques because of possible centrifugation-induced permissiveness.

In 2007, a new primary cell model was published by Sharon Lewin's group, in which they demonstrated that pre-treatment of rCD4 T cells with the chemokines CCL19 or CCL21 (the two known ligands for CCR7) enhanced permissiveness of these cells to HIV-1 infection[442]. This group subsequently demonstrated that pre-treatment with the chemokines CXCL9, CXCL10, (ligands for CXCR3) and CCL20 (ligand for CCR6) also increased permissiveness of rCD4 T cells to HIV-1 infection[456]. Importantly, it was shown that pre-treatment with these chemokines did not induce T cell activation. Similar to the O'Doherty model, this chemokine-induced model was also shown to involve changes in rapid dephosphorylation of cofilin and filamentous actin (F-actin), thereby overcoming restrictions to HIV-1 infection imposed by the actin cytoskeleton[456]. This method of infection resulted in a high percentage of rCD4 T cells becoming latently infected, although the number of cells containing integrated HIV-1 DNA was still lower than that seen in activated CD4+ T cells. It was also demonstrated that following PHA activation, these cells could produce virus. In 2011, this model was used to determine the

relevance of different LRAs in ex vivo latency reversal studies[470]. They found that, as with ex vivo studies, latency could be established without spontaneous production of virus. They also found that stimulation of latently infected rCD4 T cells with agents such as the protein kinase C (PKC) agonist, prostratin, or IL-7, closely mirrored what had been previously seen in ex vivo studies using rCD4 T cells isolated from patients on ART. These data demonstrated that this model was a useful tool for studying HIV-1 latency.

A modified version of the O'Doherty model was developed by Warner Greene and colleagues to decrease culture times and increase ease of use with reporter viruses[446]. While this model still uses spinoculation to achieve latent infection of rCD4 T cells, infection is performed with a dual-labeled reporter virus to measure both the number of cells responding to an LRA and the magnitude of the response. This model was also used to evaluate the establishment and reversal of HIV-1 latency in different resting memory CD4+ T cell subsets to study the similarities and differences in purified cell types among this heterogeneous population. This model allows for efficient screening of LRAs in less than a week, which is desirable when screening large libraries of compounds.

A fourth primary cell model of HIV-1 latency was developed by direct infection of rCD4 T cells. This model is unique in that it establishes latent infection in rCD4 T cells following a brief co-culture with autologous, productively infected cells[471]. The rCD4 T cell population is subsequently isolated from co-culture, resulting in 1-12% of cells that contain integrated HIV-1 DNA, with 0.5-5% of those cells capable of producing virus. This method establishes latent infection in all rCD4 T cell subsets, similar to that seen in vivo.

To overcome the restrictions and limitations of using a primary cell model reliant on direct infection of rCD4 T cells, several additional models have been developed that take

advantage of infecting highly susceptible, activated CD4+ T cells, followed by a transition period into a resting, latent state. There are limitations to this strategy, however, primarily because most activated CD4+ T cells die shortly after activation and cannot survive long enough to transition into a quiescent state[472]. Because these models start with activated CD4+ T cells that are then allowed to transition into a resting state, long culture times are required (on the order of 1-3 months). Increased survival times and transition from an effector to a memory state can be achieved in culture following T cell activation with the addition of cytokines, such as IL-2 and IL-7[472-475]; however, both of these cytokines have also been found to reactivate latent HIV-1[433, 476, 477]. Therefore, alternative strategies were required to overcome these limitations.

The first primary cell model of HIV-1 latency to infect activated CD4+ T cells prior to returning to a resting state, was published in 2006[443]. In this model, infected CD4+ T cells were co-cultured with the H80 feeder cell line, which was shown to promote cell survival over long-term culture in the absence of additional cytokines. Although this model resulted in high levels of latently infected cells that could be reactivated to produce virus following stimulation with prostratin, PHA, or anti-CD3/CD28, a small fraction of the resting cell population expressed the early activation marker CD69, suggesting that these cells may not be in a resting or latent state. Additionally, there were low levels of both intracellular and extracellular p24 production, indicating that some cells had ongoing viral replication. In 2010, a second model was developed that also used the H80 feeder cell line to return activated, HIV-1-infected cells to a resting state[447]. In this model, activated CD4+ T cells were infected using a VSV-G pseudotyped construct that expressed either EGFP or mCherry in place of *nef* and also contained a mutation in *tat* that had previously been shown to enhance the establishment of latency in J-lat cells[385]. These reporter genes allowed sorting of productively infected cells by fluorescence-activated cell

sorting (FACS). This method was able to generate tens to hundreds of millions of latently infected CD4+ T cells. The majority of the resulting cells were found to have a $T_{CM}$ phenotype. Much like with the previous model using the H80 feeder cell line, this model also showed low-levels of GFP expression and low-level virus production in the absence of stimulation. These cells also expressed a significant level of the activation marker CD25. Collectively, these data suggest that the H80 feeder cell line for establishing infection does not allow CD4+ T cells to return to a truly resting state.

In 2008, a model by Marini et al. was published in which they infected activated CD4+ T cells co-cultured with antigen-loaded monocyte-derived dendritic cells (MDCCs)[448]. This proof of concept study demonstrated that HIV-1 latency could be established in vitro following antigen-driven proliferation. Following co-culture and activation of $T_N$ cells and subsequent HIV-1 infection, the cells were returned to a resting state. As with the previously described model, the resulting cells were found to be almost entirely $T_{CM}$ cells. While this model used more physiological parameters to infect cells and establish latency, there was a significant decrease in cell viability. And although these cells expressed little to no CD25 or HLA-DR compared to controls, they were found to be larger and more granular than freshly isolated CD4+ T cells, which suggested that these cells might not be truly resting.

In 2009, the Bosque and Planelles model was published using a novel method to generate latently infected CD4+ T cells[445]. The authors used purified $T_N$ cells that were then stimulated with anti-CD3/CD28 in the presence of the cytokines tissue growth factor-beta (TGF-β), IL-4, and IL-2. This culturing method primed these cells into a non-polarized $T_{CM}$ phenotype, as had been previously described[478]. While the cells were activated, they were infected, via spinoculation, with a replication-defective HIV-1 clone permitting only a single round of

infection. This model was initially used to study different signaling pathways that could be targeted to reverse latency, specifically in latently infected memory CD4+ T cells. One major caveat to this model is that prior to reactivation of the latently infected cells, neither T cell activation nor viral integration were assessed, so it is unclear if the cells were truly in a resting state or if/how many cells harbored latent proviruses. A second paper published by this group extended this model to look at the effects of homeostatic proliferation, promoted by addition of the cytokines IL-2 and IL-7, on reversal of HIV-1 latency[451]. In this study, they measured both integrated HIV-1 DNA and the T cell activation marker CD25 before and after stimulation. They also assessed cellular DNA and RNA content under their culture conditions, which were found to be consistent with freshly purified rCD4 T cells. They did find, however, that a significant percentage of the $T_{CM}$ cells expressed CD25. Additionally, during culture with IL-2 and/or IL-7, there were increased levels of intracellular p24, indicating that HIV-1 was not latent.

Another model from 2009 was published by the Siliciano group, in which they took advantage of the pro-survival protein, Bcl-2[444]. Bcl-2 is an anti-apoptotic protein downstream of the IL-7 signaling pathway that prevents cell death under conditions of cytokine withdrawal[472, 479]. It had previously been shown that overexpression of Bcl-2 was able to restore peripheral T cell homeostasis in IL-7 receptor-deficient mice, demonstrating that the pro-survival benefits of IL-7 can be achieved through the use of Bcl-2, without inducing cellular proliferation or increased metabolic activity[480, 481]. Here, primary CD4+ T cells were transduced with a lentiviral vector expressing Bcl-2 and were subsequently expanded by activation using anti-CD3/CD28. Activated, transduced cells were infected with a pseudotyped, modified, reporter virus that expressed EGFP upon latency reversal. The expanded cells were allowed to return to a resting state over long-term culture, and the EGFP negative cells were sorted, providing a pure

population of rCD4 T cells, some of which were latently infected. This model provided a useful tool for long-term culture of primary cells and a reliable means to generate latently infected rCD4 T cells. The authors screened a total of 4400 compounds from two different libraries as LRAs, identifying a total of 17 compounds that were able to reactivate latent HIV-1.

While there are clear benefits to the use of activation models, there are also drawbacks. Large numbers of latently infected cells can be generated, providing an efficient platform for large-scale evaluation of LRAs. However, the resulting rCD4 T cells after in vitro activation typically do not have the same phenotype as freshly isolated rCD4 T cells, lending the question of physiological relevance. Furthermore, these models largely result in cells of a $T_{CM}$ phenotype and do not allow for evaluation of $T_N$ cells, limiting their use for T cell subset analyses.

While each primary cell model of HIV-1 latency has their strengths for studying certain aspects of latency or latency reversal, a recent report comparing LRAs in several of the models discussed above showed a high degree of variation and inconsistency between the models, none of which recapitulated the responses seen using ex vivo patient cells[471]. This report was not surprising given the vast differences between each model. Each model is unique in the type of cells isolated, treatment or modification of cells before infection, virus used for infection, and time of stimulation before readout measure (Table 1). Each model also uses a different readout method to quantify latency reversal, ranging from luciferase or GFP expression, quantification of HIV-1 mRNA, soluble RT activity in the supernatant, or infectious units per million $CD4^+$ T cells (IUPM) (Table 1). Caution should be taken when comparing results between studies on HIV-1 latency because differences seen may be due to a lack of assay standardization and not on actual biological differences.

**Table 1:** Cell models to study HIV-1 latency

| Cell Model | Type of input cells | Cell cycle at time of infection | Virus(s) used | Duration of stimulation[ρ] | Assay readout |
|---|---|---|---|---|---|
| Greene | Total or memory CD4+ T cells | Resting | NL4-3-Δnef-luciferase/GFP/mCherry | 1-3 d | Luciferase, GFP, mCherry |
| Lewin | Primary rCD4 T cells | Resting | NL4-3, AD8 | 7 d | Soluble RT activity |
| Spina | Primary CD4+ T cells | Resting | NL4-3 | 7-14 d | Tat mRNA, IC[ε] p24-gag |
| O'Doherty | Primary rCD4 T cells | Resting | IIIB | 1-3 d | IC[ε] p24-gag |
| Karn | Primary CD4+ T cells | Activated | ΔgagΔnef-tat(H13L)-IRES-EGFP | 18 h | EGFP, mCherry |
| Planelles | Naïve CD4+ T cells | Activated | Δenv or ΔenvΔnef-GFP | 7 d | IC[ε] p24-gag or % GFP+ cells |
| Cloyd | Primary CD4+ T cells | Activated | JR-CSF | 2 d | IC[ε] p24-gag |
| Marini | Activated $T_N$ and memory CD4+ T cells | Activated | IIIB | 14 d | IC[ε] p24-gag and EC[ψ] p24-gag |
| Siliciano | Primary CD4+ T cells | Activated | NL4-3-Δ6-drEGFP | 2-3 d | % GFP+ cells |
| Patient cells | Primary rCD4 T cells | NA | Endogenous | 14–21 d | Infectious units (IUPM[Υ]) |

[ρ] Indicates time between stimulation and assay readout.
[Υ] Infectious units per million cells.
[ε] Intracellular.
[ψ] Extracellular.

### 1.4.3.4 Ex vivo patient-derived cell models

The low frequency of latently infected cells in HIV-1-infected individuals on ART makes ex vivo studies using patient-derived cells a particular challenge. Latency reversal studies using ex vivo patient-derived cells usually requires either large volume blood draws or leukophereses to obtain enough cells, adding another level of complexity to these already difficult studies. Because of these limitations, ex vivo patient-derived cell models to evaluate putative LRAs have been limited.

One method using ex vivo patient-derived cells to evaluate LRAs is to treat rCD4 T cells isolated from HIV-1-infected individuals with putative LRAs prior to plating the cells for the

viral outgrowth assay, which measures the frequency of replication-competent HIV-1[471]. Comparing LRA-treated to untreated cells will determine whether or not the LRA had a specific effect on latency reversal and virus production. A modified version of this approach has been described to evaluate putative LRAs[482]. Instead of treating the patient-derived cells with the LRAs prior to co-culture with PHA-stimulated, allogeneic PBMCs for the viral outgrowth assay, treated cells are instead co-cultured with the permissive MOLT-4/CCR5 cell line that supports robust HIV-1 replication. Following co-culture, viral outgrowth was measured by extracellular p24 ELISA, and cell-associated HIV-1 RNA and DNA were measured by qPCR. This model is particularly useful because it measures virus production and replication-competent HIV-1 in addition to measuring both cell-associated HIV-1 RNA and DNA to assess the effect of each LRA has on the size of the latent reservoir.

### 1.4.4 Latency reversal as a strategy to reduce or eliminate the latent reservoir in vitro and ex vivo

Dating back to as early as 1998, it was hypothesized that if viral transcription and subsequent virus production could be induced from latently infected cells in HIV-1-infected individuals on ART, then these latently infected cells could possibly be eliminated either through cytopathic effects or through host immune effector mechanisms[483-487]. This proposed mechanism to eliminate the latent reservoir later became known as the "kick and kill" strategy (Fig. 4)[488, 489]. With elucidation of mechanisms that maintain proviral latency, attempts have been made to target or block those host factors that maintain proviral latency in the hopes of turning on viral transcription, resulting in the ultimate clearing of the now productively infected cells, and will be discussed in greater detail below.

To date, the most widely studied LRAs have been histone deacetylase inhibitors (HDACi) that target histone deacetylases. As chromatin remodeling and histone deacetylation are important for the silencing of HIV-1 transcription in rCD4 T cells[490, 491] and treatment of latently infected rCD4 T cells with HDACi were found to reverse HIV-1 latency[491, 492], at least 15 different HDACi have been evaluated for their effectiveness as LRAs to date, many of which are FDA-approved or under clinical evaluation for treatment of various cancers[493, 494]. These include: valproic acid[492, 495, 496], trichostatin A (TSA)[491, 497, 498], oxamflatin[499, 500], scriptaid[500, 501], apicidin[500, 502], sodium butyrate[495, 503], M344[500, 504], CG05/CG06[505], MC1293[506], givinostat[500, 507, 508], entinostat[497, 509], belinostat[496, 508], vorinostat (suberoylanilide hydroxamic acid, SAHA)[510-512], panobinostat (PNB)[496, 508], and romidepsin (RMD)[496, 513]. HDACi are particularly attractive

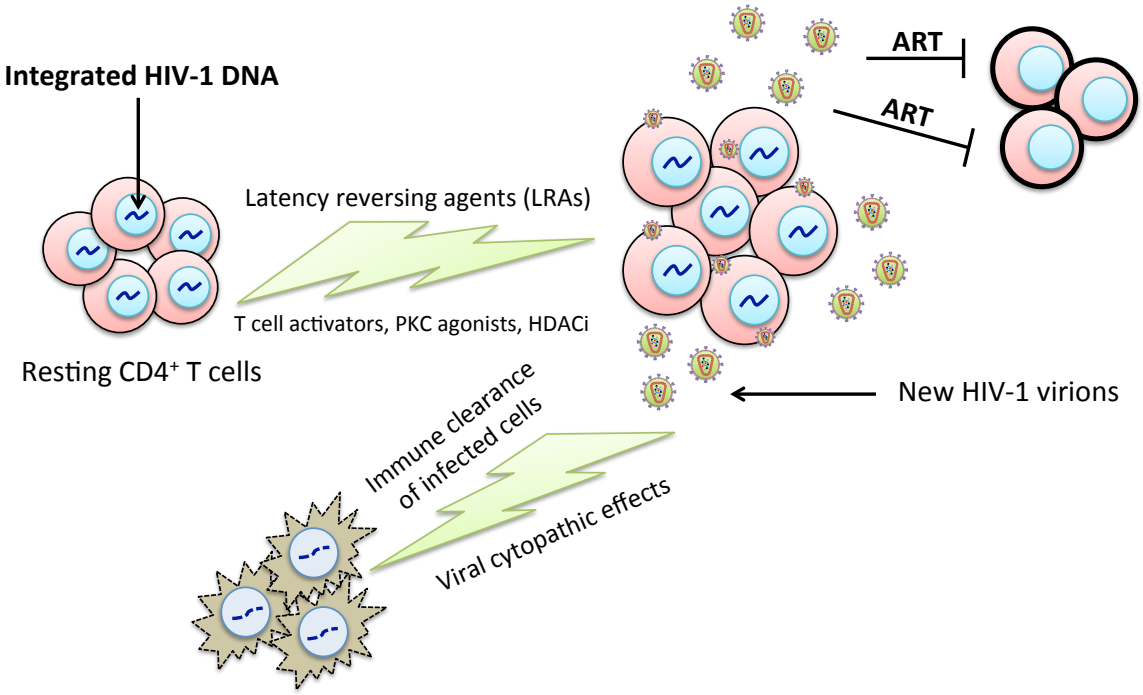



**Figure 4: "Kick and kill" strategy of latency reversal and elimination of the latent reservoir.** Latently infected rCD4 T cells are treated with an agent, such as a T cell activator or PKC agonist, to 'kick' HIV-1 out of latency and produce new virions. This is performed in the presence of ART to prevent new rounds of infection. Newly produced virions will be eliminated by the host's immune system. The producer cells will also be eliminated by the host's immune system or will be killed by viral cytopathic effects. HDACi: histone deacetylase inhibitor. Figure adapted from Garrido and Margolis, 2015[11].

compounds to assess for latency reversal potential because, in general, they have a low toxicity profile and do not induce global T cell activation[514]. While there have been too many studies to discuss individually at length, many of these HDACi proved to be ineffective at reversing latent HIV-1 or were too toxic at concentrations required to reverse latent HIV-1 and are thus no longer under investigation for clinical use. A few of the HDACi that were able to reverse HIV-1 latency, either measured by increased viral transcription or virus production, have now been evaluated clinically and will be discussed in greater detail in section 1.5.2.

Both in vitro and ex vivo evaluation of several histone methyltransferase inhibitors (HMTi) as potential LRAs have been conducted. Chaetachin[515, 516], an inhibitor of SUV39H1 (which mediates trimethylation of H3K9), BIX-01294[383, 516], an inhibitor of G9a (which mediates

dimethylation of H3K9), 3-deazaneplanocin A (DZNep)[380], a pan histone methylation inhibitor and non-specific inhibitor of EZH2, and GSK343, a selective inhibitor of EZH2, have all been evaluated as potential LRAs. Ex vivo evaluation of both chaetachin and BIX-01294 found that both of these agents potently induced virus production from rCD4 T cells isolated from HIV-1-infected individuals on ART[517]. Importantly, non-specific T cell activation was not assessed and both agents had a dose-effect on cell viability. In vitro analysis of DZNep revealed only modest effects in viral transcription when administered alone, however, there was an additive effect when combined with an HDACi, suggesting that DZNep may prime latently infected cells to be more responsive to HDACi[380]. Importantly, DZNep was cytotoxic at concentrations required to achieve latency reversal as a single agent. Ex vivo evaluation of GSK343 found similar results to those found with DZNep, such that treatment with GSK343 alone had minimal effects on reversal of proviral latency[518]. However, when cells were treated with GSK343 followed by treatment with SAHA, there were significant increases in viral transcription and virus production over treatment with SAHA alone. While there are currently no FDA-approved methyltransferase inhibitors, making their clinical evaluation in the setting of HIV-1 limited, many are currently under clinical evaluation for different cancer treatments[519].

In addition to the evaluation of HMTi, one DNA methyltransferase inhibitor (DNMTi) has also been evaluated as a potential LRA. 5-aza-2'-deoxycytidine (5-AzadC, 5-AZA-CdR, decitabine) is an FDA-approved DNMTi used to treat myelodysplastic syndromes[520]. At least three independent in vitro and ex vivo studies have evaluated the effectiveness of 5-AzadC as a potential LRA[496, 521, 522]. All three studies found that 5-AzadC was unable to substantially reactivate latent HIV-1 when used as a single agent. However, 5-AzadC did show substantial increases in virus production when combined with either an NF-κB activator[521] or an HDACi[496].

These studies suggest that while DNMTi may have limited effects on reversal of latent HIV-1, they may have synergistic effects when combined with other agents, such as NF-κB activators or an HDACi and could be further evaluated under combinatorial conditions.

Activation of the PKC pathway was one of the first suggested targets to reverse latent HIV-1. PKC is a family of serine/threonine kinases composed of several different isoforms. Activation of PKC induces activation of the ERK1/2 mitogen-activation protein kinase pathway. This pathway stimulates IKK-dependent phosphorylation of NF-κB inhibitors, the IκB proteins, via activation of IκB kinase, leading to their subsequent degradation, ultimately leading to activation and nuclear translocation of NF-κB. Once in the nucleus, NF-κB is able to mediate transcription of target genes[523, 524]. Because the HIV-1 promoter contains NF-κB binding sites, it was believed that upregulation of transcription factors such as NF-κB could activate transcription from a latent provirus.

To this effect, synthetic phorbol esters, such as PMA, natural non-tumor promoting phorbol esters, such as prostratin, non-tumor promoting lactones, such as bryostatin-1, and ingenols and ingenol derivatives, that all activate the PKC pathway have been evaluated as potential LRAs[524-534]. In general, PKC agonists have been shown to be potent activators of latent HIV-1. They consistently and reproducibly reactivate latent HIV-1 across T cell line models and primary cell models, often being used as an activation control. In addition to PKC activation, ingenol derivatives have also been shown to increase HIV-1 transcription through upregulation and activation of P-TEFb[535].

While PKC agonists are clearly potent LRAs, there are many concerns with using PKC modulation as a means to eradicate the HIV-1 latent reservoir in HIV-1-infected individuals on ART. The result of PKC activation on transcription is non-specific to HIV-1. Phorbol esters have

been shown to activate PKC-dependent transcription in multiple different cell types, raising concerns about systematic, non-specific effects, which would likely result in a detrimental outcome. In cell culture models, PKC modulation induces non-specific T cell activation and proliferation, resulting in a dose-dependent increase in cytotoxicity. Although there are no published studies reporting these results, PKC activators, mainly prostratin, have been reported to be highly toxic in NHPs at doses required to achieve a therapeutic effect on the HIV-1 latent reservoir[410].

One notable exception among the PKC agonists is bryostatin-1. Unlike the other PKC agonists, bryostatin-1 demonstrated robust in vitro latency reversal at low nanomolar concentrations without inducing T cell activation or effecting cell viability[525]. Additionally, ex vivo evaluation of putative LRAs to induce viral outgrowth from cells isolated from HIV-1-infected individuals on ART revealed that bryostatin-1 was able to significantly increase both cell-associated HIV-1 RNA and viral outgrowth while other single agents failed to do so[482, 536]. Bryostatin-1 has been evaluated clinically for non-HIV-1 related illnesses, including various malignancies and Alzheimer's disease, and has largely been found to be safe and well tolerated[537]. Given these findings, bryostatin-1 has now been evaluated clinically for the treatment of HIV-1 and will be discussed further in section 1.5.4.

Sequestration of P-TEFb prevents transcriptional elongation in rCD4 T cells. Two approaches have been made to free up P-TEFb in rCD4 T cells to reactivate latent HIV-1. Hexamethylbisacetamide (HMBA), a hybrid bipolar compound, was identified as an inducer of HIV-1 transcription in the early 1990s[538]. HMBA was later evaluated as a LRA and was found to induce viral transcription by recruiting CDK9 to the HIV-1 promoter and inducing chromatin remodeling around nuc-1[539, 540]. Importantly, this effect was found to be independent of cellular

activation or proliferation[539]. While HMBA was originally developed as an anti-cancer drug, its limited effects at clinically tolerable doses have prevented further clinical development[541]. Thus, clinical evaluation of HMBA as an anti-HIV-1 agent may be difficult.

Another agent shown to release P-TEFb from the 7SK snRNP is the BRD4 inhibitor, JQ1[542-544]. P-TEFb can be released from the 7SK snRNP either by BRD4 or by HIV-1 tat, thereby presenting BRD4 as a competitive inhibitor of P-TEFb in relation to tat[397]. JQ1 was found to be a weak activator of latent HIV-1, however, it remains of interest because it does not induce T cell activation or cellular proliferation. There are currently several bromodomain inhibitors under pre-clinical or clinical evaluation, including JQ1[545], suggesting that this drug will continue to undergo evaluation as an anti-HIV-1 agent.

Disulfiram, a compound originally identified (in the context of HIV-1) in a high-throughput screen to identify compounds that could induce HIV-1 transcription without increasing T cell activation in a primary T cell model of HIV-1 latency[546]. Disulfiram was found to significantly increase viral transcription through an unknown mechanism. It was later identified by our lab that disulfiram reactivates latent HIV-1 through depletion of the phosphatase and tensin homolog (PTEN), resulting in activation of the protein kinase B (Akt) pathway, subsequently leading to activation of NF-κB[547]. Disulfiram is an FDA-approved drug used to treat alcoholism that has been in clinical use for decades, with a well-characterized safety and tolerability profile[548-550]. Because of these findings, disulfiram has been clinically evaluated as an anti-HIV-1 agent and will be discussed in section 1.5.3.

It is important to note that there has been a great deal of variation in responses to these LRAs between studies and between models. In 2013, a detailed comparative study was published comparing the effects of 13 different LRAs or LRA combinations on latency reversal in five

different primary cell models, four T cell line models, and ex vivo patient-derived cells[471]. Strikingly, this study showed that there was an impressive inability of different LRAs to elicit an effect on latency reversal across models, and none of the cell models were able to recapitulate what was seen with ex vivo patient-derived cells. Additionally, a study from the Siliciano lab published in 2014 was designed to us an ex vivo patient-derived cell model to distinguish effective versus ineffective single agents for latency reversal[482]. This study found that no single agent that was evaluated was able to significantly or reproducibly induce viral transcription or production from rCD4 T cells isolated from HIV-1-infeted individuals on ART. The one exception was bryostatin-1, which was able to significantly increase viral transcription but not virus production. As described in section 1.4.2, control of post-integration latency in rCD4 T cells is highly dynamic and tightly regulated at many levels. All of these findings suggest that reversal of HIV-1 latency may require multiple LRAs that work synergistically to activate proviral transcription.

Several studies have now been published describing the enhanced effects of LRA combinations on latency reversal versus using single agents alone. A second study by the Siliciano lab published in 2015 found that combining PKC agonists with either HDACi or JQ1 led to a synergistic increase in latency reversal[536]. Additional studies found synergistic effects between ingenol derivatives and either JQ1[551, 552] or PNB[534], between bryostatin-1 and JQ1[551], between prostratin and JQ1[543], and between HMBA and prostratin[553]. These studies highlight the critical need of additional evaluations of not just single LRAs but LRA combinations that will likely be required to achieve a clinically significant reduction in the HIV-1 latent reservoir.

## 1.5 CLINICAL EVALUATION OF HIV-1 LATENCY REVERSAL AS A CURATIVE STRATEGY

### 1.5.1 Cytokines

The first study to describe latency reversal as a possible mechanism to reduce the latent reservoir dates back to 1998, in a publication by Chun et al[477]. Using their knowledge of HIV-1 pathogenesis and the direct role of particular cytokines, especially pro-inflammatory cytokines, in modulating HIV-1 replication, the authors tested ex vivo single or combinations of cytokines on virus production using cells from both ART-naïve and ART-treated HIV-1-infected individuals. They found that the combination of IL-6 + TNF-α + IL-2 induced virus production to similar levels as that seen from treatment with anti-CD3/CD28 in the ART-treated individuals. These findings demonstrated that modulation of the cytokine microenvironment in vivo could theoretically be used to induce latent proviruses and reduce the size of the latent reservoir.

During HIV-1 infection, it had been well documented that peripheral lymphocytes had both decreased production of and responsiveness to IL-2[554]. Therefore, both pre-clinical and clinical in vivo IL-2 administration was evaluated in HIV-1-infected individuals, largely as a means to try and increase CD4+ T cell counts and improve immune function[554-570]. While the collective findings from these early studies were largely inconclusive, showing increased CD4+ T cell counts in some with no clinical benefit in others. It quickly became clear that high-dose or continuous administration of IL-2 was associated with significant toxicity, while low-dose, intermittent administration was more tolerable. In 2000, a large retrospective study of 157 patients who participated in randomized clinical trials of IL-2 therapy to treat HIV-1 infection prior to the introduction of ART (1995) was published[571]. This study found that IL-2 treatment

led to a significant increase in CD4+ T cell counts and lower plasma HIV-1 RNA levels; however, there were no significant improvements on clinical outcome. When clinical recommendations to initiate ART were based on CD4+ T cell counts and not on diagnosis, a clinical trial was conducted to assess the effect of IL-2 on time to initiate ART based on current CD4+ T cell counts[572]. This study found that while the time to initiate ART was deferred by up to 92 weeks following IL-2 therapy, there were no significant differences in adverse or AIDS-defining events.

Based on their ex vivo preliminary findings, as well as their knowledge from previously published data on IL-2 administration for the treatment of HIV-1, Chun et al. hypothesized that intermittent low-dose administration of IL-2 to HIV-1-infected individuals on ART could induce both transient induction of HIV-1 RNA and elimination of latently infected cells through viral cytopathic effects and/or immune effector mechanisms[483-487]. This mechanism to eradicate latent HIV-1 reservoirs later became known as the "kick and kill" approach. In this study, they found that administration if IL-2 led to significant reductions in the recovery of replication-competent virus from peripheral blood CD4+ T cells. In a subset of IL-2-treated individuals, virus could not be isolated from large bulk cultures of peripheral blood CD4+ T cells or from lymph node biopsies. This led the authors to conclude that intermittent administration of low-dose IL-2 with continuous administration of ART could significantly reduce the size of the latent reservoir.

A second similar study was attempted in 3 HIV-1-infected individuals who had suppressed plasma viremia to below 5 copies/mL for at least 26 weeks[573]. In addition to administration of IL-2 while on continuous ART, they also included administration of the antibody OKT3, which is an anti-CD3-specific antibody. The idea behind this study was that IL-2 and OKT3 may work synergistically to promote T cell proliferation and increase immune

function to aid in elimination of the latent reservoir. Administration of IL-2 + OKT3 led to a significant decrease in CD4+ T cells while showing transient effects on plasma viremia and no overall effect on the size of the latent reservoir. They also found significant increases in non-specific T cell activation, cellular proliferation, and antibody-mediated immune responses against OKT3. There were also severe adverse events due to treatment, including seizures and renal failure. This finding suggested that the addition of OKT3 to IL-2 therapy was too toxic and should not be considered.

The only way to determine if the latent reservoir has been eliminated following some sort of therapeutic intervention is through treatment interruption and subsequent evaluation of viral rebound. In order to determine if intermittent low-dose IL-2 administration during continuous ART was able to eliminate latently infected cells, two individuals from the Chun et al. study, with undetectable replication-competent virus from both blood and lymph node biopsies, were enrolled in a second study to undergo a treatment interruption[574]. Following treatment interruption, both patients experienced viral rebound within 3 weeks, indicating that the latent reservoir had not been eliminated.

In 2009, a large-scale prospective study evaluating the effect of IL-2 therapy with ART on CD4+ T cell counts and clinical benefit was published[575]. This study, enrolling nearly 6,000 people between the treatment and control groups, found no clinical benefit to IL-2 treatment with ART versus ART treatment alone. They did find, however, that the addition of IL-2 treatment led to a higher frequency of grade 4 adverse events. Collectively, all of these studies have demonstrated no clinical benefit to the use of IL-2 in reducing the HIV-1 latent reservoir or long-term outcomes of disease.

Similarly to what had previously been demonstrated with the use of cytokine combinations in ex vivo cultures, IL-7 was also shown to potently induce latent HIV-1 in ex vivo cultures of purified rCD4 T cells isolated from suppressed individuals on ART[476]. Administration of IL-7 to HIV-1-infected individuals had previously shown clinical benefit through restoration of T cell homeostasis and increased thymic output[576-578]. In light of these findings, in vivo IL-7 administration was evaluated to assess its impact on latency reversal and the size of the latent reservoir[579]. While this study demonstrated that IL-7 had a minimal effect on virus production from latently infected cells, IL-7 significantly increased the size of the latent reservoir in ART-suppressed individual by increasing the total number of cells containing integrated HIV-1 DNA. This study demonstrated that the use of the homeostatic cytokine, IL-7, was not a useful therapeutic intervention for latency reversal but instead actually expanded the size of the latent reservoir in vivo.

### 1.5.2 Histone deacetylase inhibitors (HDACi)

While it became increasingly clear that the use of cytokines or non-specific T cell activation were either ineffective at reducing the latent reservoir or were found to be highly toxic, scientists searched for novel strategies that may provide better clinical benefit. In 2005, a small study evaluating the use of valproic acid plus intensified ART in 4 HIV-1-infected individuals on ART was published[580]. Valproic acid is an FDA-approved anti-convulsant and mood stabilizing drug that functions as an HDACi[581]. It had previously been shown that treatment of rCD4 T cells in vitro at clinically achievable concentrations of valproic acid did not induce T cell activation[492]. Therefore, it was speculated that the use of HDACi, such as valproic acid, could be a safe and effective way to eliminate the latent reservoir. In this study, 4 HIV-1-infected individuals

underwent ART intensification through addition of enfuvirtide plus administration of oral valproic acid. This study found a significant reduction in the size of the latent reservoir in 3 out of 4 individuals. They also found that the addition of valproic acid to ART was well tolerated. This preliminary study led to the further investigation of valproic acid as a possible treatment option to reduce or eliminate the latent reservoir.

Despite these promising initial findings, several additional small-scale studies found that administration of valproic acid to HIV-1-infected individuals on ART had limited to no effect on the size of the latent reservoir[179, 582-585]. In light of these conflicting results, a randomized multicenter, cross-over study was conducted in 56 virologically suppressed individuals who would be under study observation for up to 1 year[586]. This study conclusively found no clinical benefit to the addition of valproic acid to ART and showed no decrease in the size of the HIV-1 latent reservoir. This suggested that more potent HDACi may be needed in order to effectively reverse HIV-1 latency in vivo and reduce the size of the latent reservoir.

In 2009, it was shown that selective class 1 HDACi could potently induce virus production from both latently infected cell lines and rCD4 T cells isolated from ART-treated individuals[587]. The recently licensed class 1 HDACi, SAHA, was evaluated as an inducer of latent HIV-1 expression[510, 511]. SAHA, also known as vorinostat, is FDA-approved for treatment of cutaneous T cell lymphoma and had previously been evaluated for safety and tolerability[588, 589]. This study found that SAHA was a more potent and selective inducer of latent HIV-1 versus valproic acid, and thus provided a rationale for clinical evaluation of SAHA administration to HIV-1-infected individuals on ART.

The first clinical evaluation of SAHA administration on the HIV-1 latent reservoir was published by Archin et al. in 2012[590]. This study evaluated the effect of oral single-dose SAHA

administration on the HIV-1 latent reservoir of 8 patients who had plasma viral loads below the clinical limit of detection. While this study found an increase in cell-associated HIV-1 RNA in all 8 individuals treated with SAHA, they did not observe a reduction in the level of replication-competent virus that could be recovered from peripheral rCD4 T cells. Furthermore, changes in HIV-1 DNA were not measured so it could not be determined if SAHA treatment had any effect on the size HIV-1 DNA reservoir. One major caveat to this study was that 6 of 8 patients had low basal levels of viral transcription prior to treatment, suggesting that these cells were not truly latent. This raised the question of whether SAHA reactivated de novo viral transcription or simply increased basal levels of transcription that were already occurring. Despite the limitations of this study, it demonstrated the use of a specific therapeutic agent targeted at the HIV-1 latent reservoir that was safe and increased viral transcription from rCD4 T cells.

While this initial evaluation of SAHA used single dose administration, two additional studies evaluated the use of multi-dose[591] or daily administration[592] of SAHA on latency reversal and the size of the latent reservoir. The first study, also by Archin et al., recruited 5 study participants from the single-dose study who agreed to receive oral administration of SAHA for three consecutive days a week for 8 weeks[591]. The authors found that cell-associated HIV-1 RNA only increased in 3 of 5 patients following multi-dose administration of SAHA. Importantly, they found no significant changes in plasma HIV-1 RNA or HIV-1 DNA, indicating that multi-dose administration of SAHA did not reduce the size of the latent reservoir.

The second study enrolled 20 HIV-1-infected individuals on ART with undetectable plasma HIV-1 RNA who would receive daily single-dose SAHA for 14 consecutive days[592]. In contradiction to the previous study, this study showed statistically significant increases in cell-associated HIV-1 RNA (7.4 median fold increase) following the 14-day administration of

SAHA, however, there was a large degree of variation between donors. Aside from increases in cell-associated HIV-1 RNA, they found no significant changes in plasma HIV-1 RNA, HIV-1 DNA (total or integrated), level of inducible virus, or markers of T cell activation. These studies collectively suggest that administration of SAHA alone, either as a single dose or in multiple doses, is insufficient at reversing latent HIV-1 or reducing the latent reservoir.

In addition to SAHA, two other HDACi have been evaluated for their clinical potency as LRAs. PNB, a HDACi that is newly FDA-approved for the treatment of multiple myeloma[593], was evaluated in a phase I/II clinical study. In 2013, a comparison of multiple HDACi in phase II/III clinical evaluation for non-HIV diseases identified PNB as a potent HIV-1 LRA in a primary cell model[508]. PNB was found to significantly increase virus production from latently infected cells in comparison to SAHA, without affecting cell viability and only minimally increasing T cell activation. Therefore, clinical evaluation of PNB seemed credible.

Fifteen HIV-1-infected individuals on ART were enrolled to receive PNB for three consecutive days a week for 8 weeks in a similar design as one of the previous SAHA trials[591]. This study found a significant increase in cell-associated HIV-1 RNA (3.5 median fold increase) as well as a significant increase in plasma viral load. However, there were no significant decreases in HIV-1 DNA (total or integrated), or recovery of replication-competent virus. Nine of the 15 study participants underwent an ATI to evaluate the effect of PNB treatment on time to rebound. The median time to rebound was 17 days and was not significantly delayed from previous findings. These findings suggested that while PNB was able to effectively disrupt HIV-1 latency in vivo, it did not lead to a reduction in the HIV-1 latent reservoir or prevent or delay viral rebound following treatment interruption.

In an additional analysis of HDACi in clinical use or development revealed that the HDACi, RMD, was an even more potent LRA than PNB[513]. RMD is an FDA-approved drug used to treat T cell lymphomas[594]. A proof-of-concept phase 1b/IIa trial of 6 aviremic individuals, intravenous administration of RMD once a week for three weeks, found an increase in cell-associated HIV-1 RNA and plasma HIV-1 RNA[595]. Consistent with the previous findings from the use of other HDACi, they found that RMD did not significantly change the size of the latent reservoir or the level of replication-competent virus. Moderate increases in T cell activation were noted.

### 1.5.3 Disulfiram

In addition to the clinical evaluation of cytokines and HDACi to reduce the HIV-1 latent reservoir, the drug disulfiram has also been evaluated. This compound was originally identified (in the context of HIV-1) in a high-throughput screen to find LRAs that didn't increase T cell activation in a primary T cell model of HIV-1 latency[546]. Disulfiram is an FDA-approved drug used to treat alcoholism that has been in clinical use for decades, with a well-characterized safety and tolerability profile[548-550]. Following the identification of disulfiram as a potential LRA in an in vitro primary cell model, in was evaluated at FDA-approved clinical doses in HIV-1-infected individuals on ART for its ability to increase plasma viremia and/or decrease the size of the latent reservoir in a small phase I, single arm study. This drug was found to be safe and well tolerated. While there were no overall significant increases in plasma viremia or changes in the size of the latent reservoir, there were rapid but transient increases in plasma viremia noted in a subset of individuals that was significantly increased from baseline. This study warranted further

evaluation, possibly using higher doses or multiple doses of disulfiram to achieve clinical significance.

Following this study, a phase II dose escalation study was conducted in 30 HIV-1-infected individuals on ART[596]. This study demonstrated that doses up to 2000mg of disulfiram (4 times the FDA-approved dose) were safe and well tolerated. This study specifically assessed the impact of different doses of disulfiram on HIV-1 transcription. They found increases in cell-associated HIV-1 RNA following administration of disulfiram with all doses. No significant dose effects were seen. While this study warrants further investigation of disulfiram administration as a LRA, possibly in combination with other therapeutic interventions, the overall impact on latency reversal was limited.

### 1.5.4 PKC agonists

In pre-clinical evaluations, bryostatin-1 proved to be unique among other known PKC agonists. It induced robust in vitro and ex vivo latency reversal at low nanomolar concentrations without inducing T cell activation or effecting cell viability[482, 525, 536]. These collective findings led to the clinical evaluation of bryostatin-1 as a LRA in a phase I pilot study[597].

Much like many of the other LRAs under clinical evaluation for the treatment of HIV-1, bryostatin-1 has been evaluated in several phase I and II clinical trials as an antineoplastic agent to treat various cancers[598]. In this pilot study, 12 participants were enrolled and divided into two arms receiving different doses (10 vs 20 $\mu g/m^2$) of bryostatin-1. They measured both cell-associated and plasma HIV-1 RNA as well as PKC activity and biomarkers of inflammation. Much like previous trials, this study found that bryostatin-1 was safe and well tolerated at both concentrations tested, however, there was no increase in cell-associated or plasma HIV-1 RNA.

There were also no detectable increases in PKC activity or in biomarkers of activation. These findings suggest that the concentrations of bryostatin-1 used in this study to reverse latent HIV-1 were likely not high enough to achieve a clinical effect. Further studies are required to determine if higher doses of bryostatin-1 are safe and if they are able to reverse HIV-1 latency and reduce the size of the latent reservoir.

The collective findings thus far from clinical evaluations of LRAs have demonstrated that while some agents are able to provide a "kick," in terms of either increased viral transcription or virus production from latently infected cells, they are not effective at inducing a "kill," as measured by no change in the size of the HIV-1 DNA reservoir or recovery of replication-competent virus. Because of their known clinical recommendations, the LRAs that have been tested clinically to target the HIV-1 latent reservoir have been found to be safe and well tolerated, with minimal increases in adverse events over ART treatment alone. These findings provide hope that putative LRAs, such as the HDACi, may be used clinically in combination with other agents that are able to efficiently aid in the kill of latently infected cells to reduce or eliminate the HIV-1 latent reservoir in infected individuals on ART. A further evaluation of the HIV-1 latent reservoir, including a better understanding of HIV-1 latency in the individual subsets of CD4+ T cells that constitute the latent reservoir, will help provide useful insight into the mechanisms or pathways that may need to be exploited to significantly reduce the size of the latent reservoir.

# 2.0 SPECIFIC AIMS

The presence of an HIV-1 latent viral reservoir in rCD4 T cells[155-157], established shortly after infection[370], presents a stable mechanism for life-long persistent infection[4, 159]. rCD4 T cells are a heterogeneous population consisting of $T_N$, $T_{SCM}$, $T_{CM}$, $T_{TM}$, $T_{EM}$, and $T_{TD}$ cells, all of which have been implicated as reservoirs of latent HIV-1[599]. A preliminary evaluation of the contribution of each CD4+ T cell subset to the HIV-1 latent reservoir in HIV-1-infected individuals on ART revealed that the $T_{CM}$ cells, followed by the $T_{TM}$ cells, were the major contributing cell subsets to the latent reservoir, based on both the frequency of each subset in the peripheral blood and the frequency of HIV-1 DNA[317]. This study also found that $T_N$ cells were only a minor contributor to the latent viral reservoir and were regarded as insignificant. As such, the vast majority of studies evaluating HIV-1 latency and latency reversal have focused on resting memory cells as a whole or in purified $T_{CM}$ cells, largely omitting $T_N$ cells from further evaluation. **Therefore, we propose to investigate the establishment and reversal of HIV-1 latency in purified $T_N$ and $T_{CM}$ cells using both an in vitro primary cell model as well as in ex vivo patient-derived cells.**

Based on this previously published data, we hypothesize that both the establishment and reversal of latent HIV-1 would differ between the different subsets of rCD4 T cells that constitute the latent reservoir. As such, we would expect the efficiency of the "kick and kill" approach to be different in the different CD4+ T cell subsets. This would suggest that more than

one approach may be needed to efficiently eliminate all infected cells within the latent reservoir in HIV-1-infected individuals on ART.

**AIM 1: Develop a primary cell model of HIV-1 latency in purified $CD4^+$ $T_N$ and $T_{CM}$ cells.**

a. Determine the efficiency of virus production from $T_N$ and $T_{CM}$ cells following latency reversal with a variety of LRAs that target different signaling pathways or repressive transcription mechanisms.
b. Determine how CCL19 makes rCD4 T cells permissive to HIV-1 infection.

The limited evaluation of $T_N$ cells has largely demonstrated that virus production following latency reversal is limited when assessed on a population level[317, 360]. However, one study revealed similar levels of virus production between $T_N$ cells and $T_{CM}$, $T_{TM}$, or $T_{EM}$ cells following latency reversal when normalized for differences in infection frequency[600]. We hypothesize that $T_N$ cells will produce similar levels of virus compared to $T_{CM}$ cells following latency reversal when using rigorous, ultrasensitive quantitative evaluations to measure low-level virus production and normalize for differences in infection frequency.

**AIM 2: Determine if the data obtained using our primary cell model of HIV-1 latency in AIM 1 are reproducible in purified $T_N$ and $T_{CM}$ cells from HIV-1-infected individuals on long-term ART.**

a. Determine if $T_N$ cells produce similar levels of virus compared to $T_{CM}$ cells when normalized for differences in infection using a variety of LRAs.
b. Determine the frequency of replication-competent virus recovered from $T_N$ and $T_{CM}$ cells.

Using the same quantitative analyses as used in AIM 1, we hypothesize that we would also see similar levels of virus production from $T_N$ and $T_{CM}$ cells following latency reversal using cells isolated from HIV-1-infected individuals on ART. While there are currently conflicting views in the field on using PCR-based quantitative measures as a surrogate marker for cell culture-based quantitative measures of latency reversal[441, 601], we would expect to see similar levels of replication-competent virus recovered from $T_N$ and $T_{CM}$ cells using the quantitative viral outgrowth assay, if total virus production (measured by extracellular HIV-1 RNA) is a surrogate measure for replication-competent virus.

**AIM 3: Determine if specific ARVs or ARV drug classes affect virus production in latency reversal strategies using a primary cell model of HIV-1 latency.**

Previous publications have demonstrated that NNRTIs are able to prevent virus production in HIV-1-transfected transformed cell lines through increased intracellular processing of Gag and Gag-Pol and premature activation of HIV-1 protease[602, 603]. Based on these findings, we hypothesize that NNRTIs will decrease virus production compared to other ARV drug classes when employing the "kick and kill" strategy of latency reversal.

# 3.0 MATERIALS AND METHODS

*Purification of CD4+ T cell subsets from HIV negative donors*

Blood was either obtained as a 180mL whole blood large volume blood draw or a buffy coat obtained from the Central Blood Bank, which were approved by the University of Pittsburgh Institutional Review Board. Written informed consent was provided for all donors. PBMCs were isolated by Ficoll-Paque$^{TM}$ PLUS (GE Healthcare) density gradient centrifugation. rCD4 T cells were purified by first isolating total CD4+ T cells by magnetic bead negative selection using a CD4+ T cell purification kit, followed by magnetic bead negative selection using anti-CD25, anti-CD69, and anti-HLA-DR antibodies. $T_N$ cells were isolated from the rCD4 T cells by magnetic bead depletion of CD45RO+ cells. $T_{CM}$ cells were isolated from the rCD4 T cells by magnetic bead depletion of CD45RA+ cells, followed by positive selection of CCR7 expressing cells. Increased labeling times were used to increase cell purity. All magnetic bead purification kits and antibodies were obtained from Miltenyi Biotec. The purity of the $T_N$ and $T_{CM}$ cells was assessed by flow cytometry (LSR II, BD Biosciences) using the following antibodies: CD3-V450, CD4-PerCP-Cy5.5, CD45RA-FITC, CCR7-PE, CD27-APC-H7, and CD62L-APC (BD Biosciences). Data were analyzed using FlowJo vX.0.7. The $T_N$ and $T_{CM}$ cell surface phenotypes were as follows: $T_N$ cells (CD45RA+, CCR7+, CD27+, CD62L+) and $T_{CM}$ cells (CD45RA-, CCR7+, CD27+, CD62L+). CD4+ $T_N$ and $T_{CM}$ cell purity was routinely found to be $\geq$ 98 % and $\geq$ 96 %, respectively (see Fig. 6A).

*Purification of CD4+ T cell subsets from HIV-1+ leukophereses*

Leukophereses were obtained from long-term suppressed HIV-1+ individuals following written informed consent and approval by the University of Pittsburgh Institutional Review Board. Purification of rCD4 T cells was performed the same as described above; however, we also saved cell pellets of total CD4+ T cells and the CD25+CD69+HLA-DR+ activated T cells for DNA quantification. CD4+ $T_N$ cells were purified from the rCD4 T cells first by removing cells that expressed CD45RO, yielding the total naïve cell fraction, followed by removal of cells expressing CD95, splitting this fraction into 'true' $T_N$ cells and $T_{SEM}$ cells. The $T_{CM}$ cells were purified from the rCD4 T cells first by removing cells that expressed CD45RA followed by positive selection for cells expressing CD62L. The CD62L- fraction was also saved as the $T_{TM}$+$T_{EM}$ cell population for DNA quantification and additional experiments. A detailed schematic outlining the purification process and all CD4+ T cell subsets isolated is shown in Fig. 5.

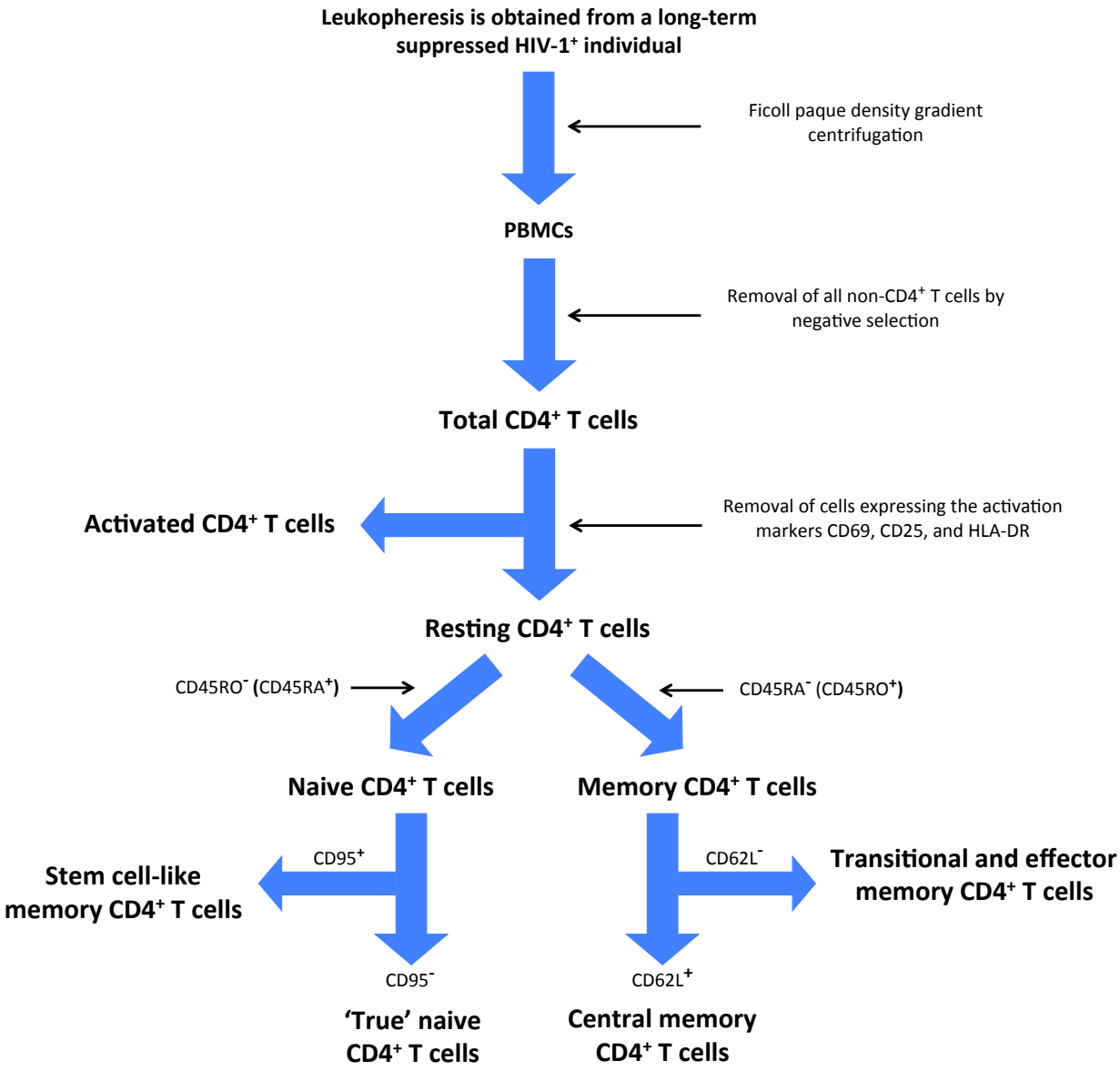



**Figure 5: Schematic representation of strategy utilized to purify CD4+ T cell subsets from Leukaphereses.**

*Infection of primary CD4+ T cell subsets*

Purified resting $T_N$ or $T_{CM}$ cells were cultured at a density of 1-2 x $10^6$ cells/mL in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 µg/mL streptomycin and 0.29 mg/mL glutamine (all from Life Technologies). CCL19 (100 nM final concentration) was added to the cells 2 days prior to infection with HIV-1, as described previously[604]. Cells were infected with either the CXCR4-tropic strain HIV-$1_{LAI}$[605] or the CCR5-tropic strain HIV-$1_{BaL}$ at a multiplicity of infection (MOI) of 1 (as titered on GHOST cells[606]) for 2 to 3 h at 37°C. HIV-$1_{BaL}$ was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: HIV-$1_{BaL}$ from Drs. Gartner, Popovic and Gallo[607]. Cells were then washed twice with fresh media to remove free virus. Every 2 days following infection 10 units/mL recombinant IL-2 (Roche) was added to the media, in addition to 300 nM efavirenz (EFV, NIH AIDS Reagent Program) to inhibit multiple rounds of HIV-1 infection. For some experiments where noted, 300 nm RAL (NIH AIDS Reagent Program), was also included to block multiple rounds of HIV-1 infection.

*Flow Cytometry*

T cell activation was assessed by flow cytometry using the following antibodies from BD Biosciences: CD3-V450, CD4-PerCP-Cy5.5, CD25-PE-Cy7, CD69-PE, and HLA-DR-FITC. To measure the expression of the HIV-1 co-receptors CCR5 and CXCR4, $T_N$ and $T_{CM}$ cells were stained with CD3-V450, CD4-PerCP-Cy5.5 and either CCR5-PE or CXCR4-PE (BD Biosciences). Typically 50,000-100,000 cells were collected per sample in the CD3+CD4+ gate to adequately measure CCR5 or CXCR4 expression. Dead cells were excluded based on SSC-A and FSC-A plots. For some experiments where noted in the text, cell viability was determined

using a LIVE/DEAD fixable cell viability dye for flow cytometry (Invitrogen). The intracellular proliferation marker, Ki-67, was stained following the manufacturer's protocol (BD Biosciences); however, instead of using the cell viability solution (7AAD) to discriminate live cells from dead cells, the cells were first stained with LIVE/DEAD-APC (Invitrogen) prior to fixation and permeabilization for Ki-67 staining. All samples were run on an LSRII and the data were analyzed using FlowJo vX.0.7.

*Reactivation of latent HIV-1 from $T_N$ and $T_{CM}$ CD4+ T cells following in vitro infection*

Seven days post-infection, the $T_N$ and $T_{CM}$ cells were washed with media and plated in a 96-well plate at a density of 100,000 cells/well. Anti-CD3/CD28 antibodies (3 beads per cell; Life Technologies), 10 nM PMA (Sigma Aldrich) + 10 µg/mL PHA (Remel), 5 µM prostratin, or 500 nM SAHA (Cayman Chemicals) was then added to duplicate wells. Unstimulated cells were used as a control. Three and 7 days post-stimulation, IL-2 (10U/mL) and EFV (300nM) were added to each well.

*Reactivation of latent HIV-1 from $T_N$ and $T_{CM}$ cells isolated from HIV-1-infected donors*

Following purification, $T_N$ and $T_{CM}$ cells were plated in separate 24-well plates at a density of $10^6$ cells/well. Cells were cultured in the presence of 300 nM EFV + 300 nM RAL to prevent viral spread, in addition to 10 U/mL IL-2 to maintain cell viability. Cells were stimulated in duplicate wells with anti-CD3/CD28 antibodies (3 beads per cell; Life Technologies), 10 µg/mL PHA (Remel) + 100 U/mL IL-2 (Roche), 5 nM PMA + 500 µg/mL ionomycin (Sigma), 5 µM prostratin, 17 nM PNB (pulsed for 30 minutes, Selleckchem), or 50 nM RMD (pulsed for 4

hours, Selleckchem). Unstimulated cells were used as a control. Two and four days post-stimulation, IL-2 (10U/mL), EFV (300nM), and RAL (300nM) were added to each well.

*Extraction and quantification of HIV-1 DNA*

Total cellular DNA was extracted from pooled duplicate culture wells, and was assayed for total HIV-1 DNA and 2-LTR circle DNA levels by qPCR, as described previously[183, 235]. Each sample was run in triplicate using the LightCycler® 480 System (Roche). DNA standards were included as described previously[183, 235]. HIV-1 DNA and 2-LTR circles were normalized to the total number of cells assayed by qPCR amplification of the *CCR5* gene[608].

*Extraction and quantification of extracellular virion-associated HIV-1 RNA*

Culture supernatant was centrifuged at 16,100 x g for 70 min to pellet HIV-1 virions. Viral RNA was then extracted using the RNeasy PLUS Mini Kit (Qiagen), and quantified using a real-time reverse transcriptase (RT)-initiated PCR assay with single-copy sensitivity, as described previously[160] using AffinityScript Multiple Temperature RT (Agilent technologies) in place of Superscript II RT. The primers and probe used to quantify HIV-1 RNA were the same as those used to quantify total HIV-1 DNA. No RT control wells were run for each sample to ensure amplification was from RNA only and not DNA.

*Calculating decay of HIV-1-infected cells following latency reversal*

HIV-1 DNA was quantified by qPCR and normalized to cell number as described above at each respective time point following latency reversal with anti-CD3/CD28, PMA + PHA, prostratin, and SAHA. The level of HIV-1 DNA/cell in unstimulated control cells was normalized to 100

for each donor. The level of HIV-1 DNA/cell following treatment with each LRA was then normalized to the level of HIV-1 DNA/cell from the unstimulated control cells. The $\log_{10}$ of each value was then plotted on a linear scale to generate linear regression curves and decay rates.

*Integration site sequencing*

Genomic DNA (20 μg) was isolated using the DNeasy Blood and Tissue Kit (Qiagen) from resting $T_N$, $T_{CM}$, and PHA-activated CD4+ T cells infected with HIV-$1_{LAI}$. DNA was digested overnight with 100 U each of MseI and BglII and purified using the QIAquick PCR Purification Kit (Qiagen). Double-stranded asymmetric linkers were prepared by heating 10 μM of each oligonucleotide to 90°C in 10 mM Tris–HCl, pH 8.0 and 0.1 mM EDTA and slowly cooled to room temperature. Linker DNA (1.5 μM) was ligated with digested cellular DNA (1 μg) overnight at 12°C with 800 U T4 DNA ligase in four parallel reactions, and the DNAs were pooled and re-purified using the QIAquick PCR Purification Kit. Semi-nested PCR was used to selectively amplify integration sites, with reactions multiplexed into eight separate samples per PCR stage. The first and second rounds of PCR utilized nested HIV-1 U5 primers, whereas the same linker-specific primer was used for both rounds. The linker primer and second round U5 primer each encoded adapter sequences necessary for Illumina sequencing, as well as sequencing primer binding sites. To afford the identification of unique library samples from multiplexed sequencing runs, unique bar-coded linker DNAs and linker-specific primers were employed for each sample, and the nested U5 primer additionally encoded a unique 6 bp index sequence. The sequences of utilized oligonucleotides are shown in Table 2. Each PCR contained 100 ng template DNA, 1.9 μM U5 primer and 0.375 μM linker primer. Each reaction was carried out using the Advantage 2 Polymerase Mix (Clontech) according to the manufacturer's instructions,

and incubated at 94°C for 2 min, followed by 30 cycles at 94°C for 15s, 55°C for 30s, and 68°C for 45s, which was followed by a final extension for 10 min at 68°C. Pooled PCRs were purified using the QIAquick PCR Purification Kit, and second round reactions were submitted for sequencing on the Illumina MiSeq platform at the Dana-Farber Cancer Institute Molecular Biology Core Facilities. Resulting sequences were mapped to the hg19 version of the human genome using BLAT, allowing for a minimum of 97 % unique sequence identity match. Correlations of integration site distributions relative to various genomic features were conducted using BEDTools[609]. Statistical analysis of resulting integration frequencies was determined using R[610, 611], with statistical significance being calculated by Fisher's exact test and Wilcoxon rank sum test.

**Table 2:** Oligonucleotide sequences used for integration site mapping.

| Oligonucleotide Use | Oligonucleotide Sequence (5'-3') |
|---|---|
| First Round HIV-1 LTR Primer | TGTGACTCTGGTAACTAGAGATCCCTC |
| Linker Top Strand (Activated CD4+) | $PO_4$-GTCCCTTAAGCGGAG-$NH_2$ |
| Linker Bottom Strand (Activated CD4+) | GTAATACGACTCACTATAGGGCCTCCGCTTAAGGGACT |
| Linker Primer (Activated CD4+) | CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCT TCCGATCTGTAATACGACTCACTATAGGGC |
| HIV-1 LTR Primer (Activated CD4+) | AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCG ATCTCGATGTGAGATCCCTCAGACCCTTTTAGTCAG |
| Linker Top Strand (CD4+ $T_N$) | $PO_4$-CGAGGCGTCTAATGC-$NH_2$ |
| Linker Bottom Strand (CD4+ $T_N$) | GCTATAGCAGCACATCAGTTAGGCATTAGACGCCTCGT |
| Linker Primer (CD4+ $T_N$) | CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCT TCCGATCTGCTATAGCAGCACATCAGTTAG |
| HIV-1 LTR Primer (CD4+ $T_N$) | AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCG ATCTTGACCAGAGATCCCTCAGACCCTTTTAGTCAG |
| Linker Top Strand (CD4+ $T_{CM}$) | $PO_4$-CTATGACGGTGACGC-$NH_2$ |
| Linker Bottom Strand (CD4+ $T_{CM}$) | GAGAATCCATGAGTATGCTCACGCGTCACCGTCATAGT |
| Linker Primer (CD4+ $T_{CM}$) | CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCT TCCGATCTGAGAATCCATGAGTATGCTCAC |
| HIV-1 LTR Primer (CD4+ $T_{CM}$) | AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCG ATCTACAGTGGAGATCCCTCAGACCCTTTTAGTCAG |

*Quantification of F-actin density*

F-actin density was quantified both by flow cytometry and confocal microscopy. For flow cytometry, cells were first surface stained for CD3-V450 and CD4-PerCP-Cy5.5 (BD Biosciences), then fixed in 1% paraformaldehyde (PFA), permeabilized with 0.1% Triton-X (Sigma Aldrich) supplemented with 5% FBS, and then stained with 100 nM phalloidin-FITC (Sigma Aldrich) in phosphate-buffered saline (PBS) supplemented with 5% FBS. Following staining, cells were washed with PBS supplemented with 5% FBS to remove any unbound phalloidin. All samples were run within 24 h on an LSRII and the data were analyzed using FlowJo vX.0.7. 10,000 events were collected per sample in the CD3+CD4+ gate. Dead cells were excluded based on SSC-A and FSC-A plots. Where specified, latrunculin A (Thermo Fisher) was added to cells 6 h prior to the addition of CCL19. Forty-eight hours post-CCL19 treatment, cells were analyzed for F-actin density and cell viability by flow cytometry as described above. For imaging, cells were fixed in 1% PFA, permeabilized with 0.1% Triton-X supplemented with 5% FBS, washed with PBS supplemented with 5% FBS, and then stained with 1U/mL phalloidin-AF488 (Life Technologies) in PBS supplemented with 5% FBS. The cells were washed, stained with 1µg/mL 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies ), and washed again. After the second wash, the cell pellets were resuspended in gelvatol mounting media and mounted onto microscope slides for imaging. Images were collected using a Nikon A1 R spectral confocal microscope with a 100x 1.4NA oil immersion objective. Following collection using NIS-Elements images were parsed to Imaris (Bitplane) and rendered in three-dimensions. The total actin volume in each cell was calculated following surface extraction and volume rendering in Imaris.

*Quantification of cellular dNTP concentrations*

Cellular dNTPs were extracted from 5-8 million cells and quantified as described previously[612].

*Quantification of replication-competent virus*

Replication-competent virus was measured using the quantitative viral outgrowth assay as previously described[613]. Duplicate wells were cultured in five-fold dilutions starting with one million cells per well down to 320 cells per well. Before PHA-activated lymphoblasts were added to each well, they were CD8-depleted by positive selection using CD8 microbeads (Miltenyi Biotec). The infectious units per million cells were calculated as described previously[613, 614]. The frequency of cultures harboring replication-competent virus was calculated by dividing the total number of wells that were p24 positive by the total number of wells that were cultured.

*Cell lines*

J89GFP cells[615] and jurkat cells (clone E6-1, ATCC® TIB-152™) were cultured at a density of 1-2 x $10^6$ cells/mL in RPMI-1640 supplemented with 10% FBS, 100 units/mL penicillin, 100 µg/mL streptomycin, and 0.29 mg/mL glutamine (all from Life Technologies). Prior to treatment with ARVs (EFV, 1-10µM; RPV, 1-10µM; 3TC, 50µM, 250µM; TNV, 100µM, 500µM; all from the AIDS Reagent Repository), cells were plated in 96 well plates at a density of 200,000 cells/well. ARVs were added to triplicate wells prior to stimulation with 30nM PMA (Sigma Aldrich). Both stimulated and unstimulated jurkat cells were used as negative controls. Unstimulated J89GFP cells were used as a background control. To assess viral transcription, the cells were harvested 48 h post-stimulation and first stained for cell viability by LIVE/DEAD-

APC staining (Invitrogen), and then run on an LSRII to measure both cell viability and GFP expression. The culture supernatant was used to measure virus production by p24 ELISA according to the manufacturer's instructions (ZeptoMetrix Corporation).

*Statistical analyses*

Statistical analysis of integration sites was determined by Fisher's exact test or Wilcoxon rank sum test (Supplemental Table 1). Statistical comparison between paired samples was analyzed using a Wilcoxin matched-pairs signed rank test for nonparametric analyses or a student t test for parametric analyses. For all unpaired samples in which the distribution was either unknown or was not normally distributed, p values were determined using a Mann-Whitney test. Spearman correlation analyses were used to determine significant correlations between virion production and the frequency of HIV-1 infection post-treatment with LRAs. For all statistical analyses, a p value $< 0.05$ was considered significant.

# 4.0 ESTABLISHMENT AND REVERSAL OF HIV-1 LATENCY IN PRIMARY CD4+ NAÏVE AND CENTRAL MEMORY T CELLS IN VITRO



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Author contribution: J.M.Z. performed all cell culture experiments, HIV-1 RNA and DNA quantifications, flow cytometry, and statistical analyses. J.M.Z. and N.S.C. conceived of the study and drafted the manuscript. All other authors read and approved of the manuscript. E.S.

and A.N.E. performed integration site analysis on extracted DNA samples provided by J.M.Z. G.L. and B.K. quantified cellular dNTPs purified and provided by J.M.Z. S.C.W. performed confocal microscopy and actin quantification on cells that were stained, mounted, and provided by J.M.Z. Z.A. helped stain and mount cells for actin imaging and quantification. Z.A. also contributed intellectually and technically to experiments.

## 4.1 INTRODUCTION

The rCD4 T cell population is heterogeneous and includes $T_N$, $T_{CM}$, $T_{TM}$, $T_{EM}$, and $T_{TD}$ cells. In HIV-1-infected individuals, viral DNA has been detected in all of these CD4+ T cell subsets, although the levels of total HIV-1 DNA are typically highest in the $T_{CM}$ and $T_{TM}$ compartments, suggesting that they may be the major reservoirs of latent viral infection[317, 360, 362, 600]. The quantitative viral outgrowth assay (QVOA), which measures the frequency of replication-competent HIV-1, has long been considered a gold standard measure of the size of the inducible latent reservoir in individuals on suppressive ART[613]. The frequency of replication-competent HIV-1 has shown a great deal of variation between studies and is dependent on how long the patient population has been on therapy and if they were treated during chronic or acute infection[371, 441, 616]. Replication-competent virus in cells from infected individuals on suppressive ART has also been recovered in all of the different T cell subsets[317, 340], albeit to varying degrees due to donor variation. One study revealed that while replication-competent HIV-1 was consistently detected within $T_{CM}$ cells, it was not frequently detected in the $T_{TM}$ cell compartment[616]. They further showed that more infectious virus could be recovered from $T_N$ cells than $T_{TM}$ cells when individuals were treated during chronic versus acute HIV-1

infection[616]. These findings highlight that quantification of viral DNA alone is not necessarily predictive of the size of the inducible latent reservoir, and suggests caution in labeling a cellular reservoir of latent HIV-1 as "major" based solely on the frequency of infection.

In addition to the memory CD4+ T cell subsets, HIV-1 DNA is almost always detected in $T_N$ cells in both viremic and suppressed individuals, although with a much lower frequency compared to the $T_{CM}$ and $T_{TM}$ compartments[309-317, 340, 600, 617-619]. Interestingly, in 2013 Saez-Cirion et al. reported that in some HIV-1-infected individuals who received ART within 10 weeks of primary infection, viremia could be controlled for at least 24 months post-treatment interruption[360]. In this patient population, HIV-1 DNA was only detected in CD4+ $T_N$ cells in 2 of 11 individuals, whereas all the resting memory CD4+ T cell subsets ($T_{CM}$, $T_{TM}$ and $T_{EM}$) harbored comparable levels of HIV-1 DNA[360]. This finding suggests that the latent HIV-1 reservoir in CD4+ $T_N$ cells may be more important than previously considered.

The naïve and different resting memory CD4+ T cell subsets differ in lifespan, proliferative capacity, antigen response time, residence throughout the body, and in their HIV-1 co-receptor, CCR5 and CXCR4, expression levels[355, 620, 621]. In light of this, we hypothesized that the establishment and reversal of HIV-1 latency would differ between naïve and memory CD4+ T cells, and that understanding these phenotypes in different CD4+ T cell subsets could facilitate the development of effective cure strategies to purge the latent reservoir. Given the low frequency of latently infected cells in ART-suppressed individuals, approximately 100 copies of HIV-1 DNA or one infectious unit per $10^6$ rCD4 T cells[158, 440, 441], we sought to compare and contrast latent HIV-1 infection using a primary cell model in highly purified $T_N$ and $T_{CM}$ CD4+ T cells.

## 4.2 RESULTS

### 4.2.1 Direct Infection of rCD4 Naïve and Central Memory T Cells With HIV-1

Given the low infection frequency of HIV-1 in individuals on ART, it is difficult to use donor-derived cells to perform detailed in vitro analyses. Therefore, we established an appropriate in vitro primary cell model of HIV-1 latency in rCD4 $T_N$ and $T_{CM}$ cells. We chose to expand the assay system developed by Saleh et al., which uses the chemokine CCL19 to enhance the permissiveness of rCD4 T cells to HIV-1 infection, thereby maintaining the integrity and authenticity of the $T_N$ and $T_{CM}$ cell populations without inducing T cell activation or differentiation[442]. Because prior published studies using this model had only characterized the establishment and reversal of HIV-1 latency in total rCD4 T cells[442, 456, 470], we first quantified the ability of X4- (HIV-$1_{LAI}$) and R5-tropic (HIV-$1_{BaL}$) strains of HIV-1 to infect highly purified CD4+ $T_N$ and $T_{CM}$ cells (Fig. 6A) in the absence and presence of CCL19 (Fig. 6C, 6D). Both cell types were equally resistant to HIV-$1_{LAI}$ and HIV-$1_{BaL}$ infection in the absence of CCL19 (Fig. 6C, 6D). We found that CCL19 significantly enhanced HIV-$1_{LAI}$ infection of both $T_N$ (Fig. 6C) and $T_{CM}$ (Fig. 6D) cells as assessed by quantification of total HIV-1 DNA. However, $T_{CM}$ cells were more efficiently infected (mean fold increase = 15.5) than were the $T_N$ cells (mean fold increase = 3.65). CCL19 was also found to increase the ability of HIV-$1_{BaL}$ to infect $T_{CM}$, but not $T_N$, cells (Fig. 6C, 6D). The latter could be due to the extremely low surface expression of CCR5 on $T_N$ cells, which was not affected by exposure to CCL19 (Fig. 7). Treatment with CCL19 also had no affect on the surface expression of CCR5 on $T_{CM}$ cells or CXCR4 expression on either cell type (Fig. 7). A small percentage of HIV-1 reverse transcripts that fail to integrate are converted to two long terminal repeat (2-LTR)-containing circles[622].

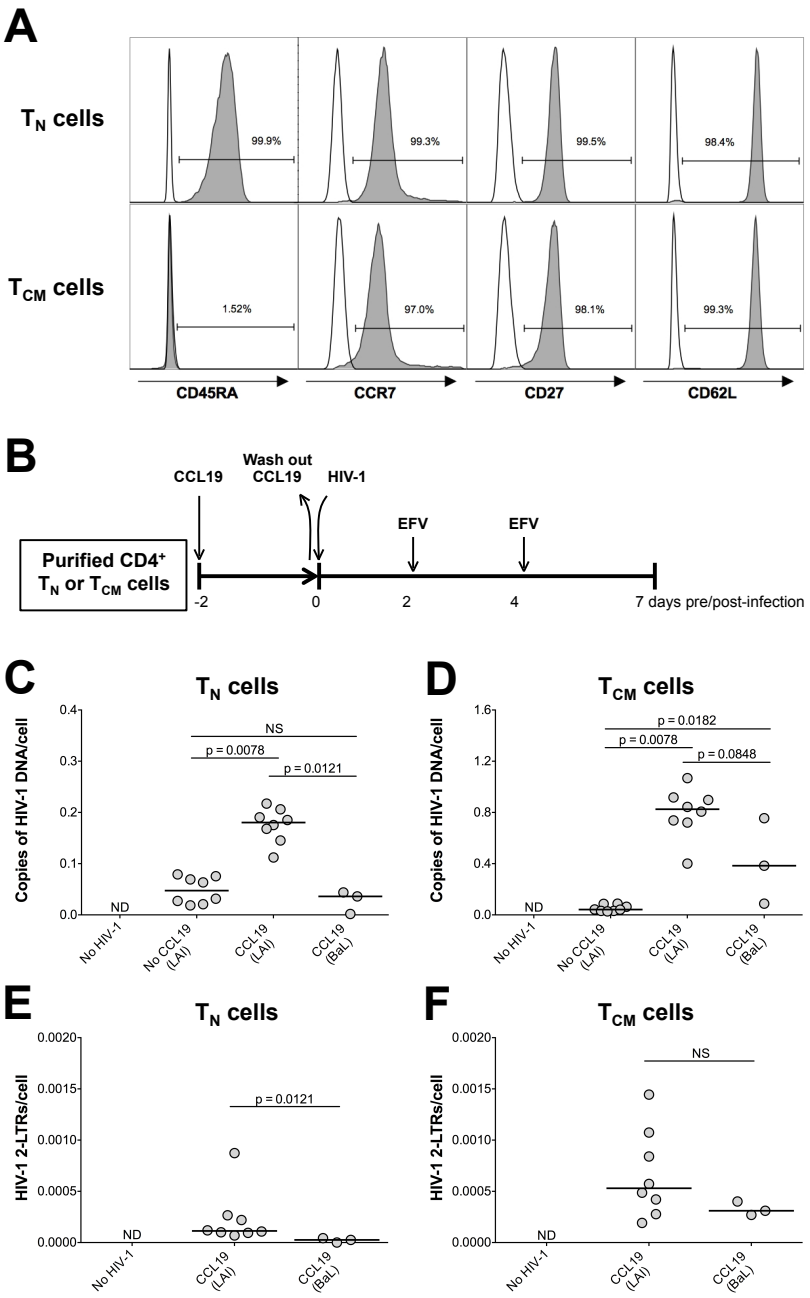


**Figure 6: Infection of $T_N$ and $T_{CM}$ cells by CXCR4-tropic (HIV-$1_{LAI}$) and CCR5-tropic (HIV-$1_{BaL}$) HIV-1 in the absence and presence of CCL19.** (**A**) $T_N$ and $T_{CM}$ cells were purified from rCD4 T cells based on the variable cell surface expression of CD45RA, CCR7, CD27 and CD62L. $T_N$ cells were defined as $CD45RA^+/CCR7^+/CD27^+/CD62L^+$. $T_{CM}$ cells were defined as $CD45RA^-/CCR7^+/CD27^+/CD62L^+$. The purity of each subset was determined by surface expression of each marker as measured by flow cytometry. Representative histograms from one donor are shown. (**B**) Schematic representation of the experimental approach. (**C**) Quantification of total HIV-1 DNA in $T_N$ cells 7 days post-infection. (**D**) Quantification of total HIV-1 DNA in $T_{CM}$ cells 7 days post-infection. (**E**) Quantification of HIV-1 2-LTR circles in $T_N$ cells 7 days post-infection. (**F**) Quantification of HIV-1 2-LTR circles in $T_{CM}$ cells 7 days post-infection. For graphs in (**C**-**F**), each dot represents a unique donor and data are normalized to cell number by qPCR quantification of the *CCR5* gene. ND = not detected. P values between No CCL19 (LAI) and CCL19 (LAI) were calculated using a Wilcoxin matched-pairs signed rank test. P values between LAI and BaL were calculated using a Mann-Whitney test.

Accordingly, we quantified HIV-1 2-LTR circle levels as a surrogate for unintegrated HIV-1 DNA (Fig. 6E, 6F). As expected, this analysis revealed that 2-LTR circles constituted only a minor proportion of the total intracellular HIV-1 DNA of both subsets. Moreover, the relative levels of 2-LTR circle DNA mimicked total HIV-1 levels across the different conditions of virus infection (Fig. 6).

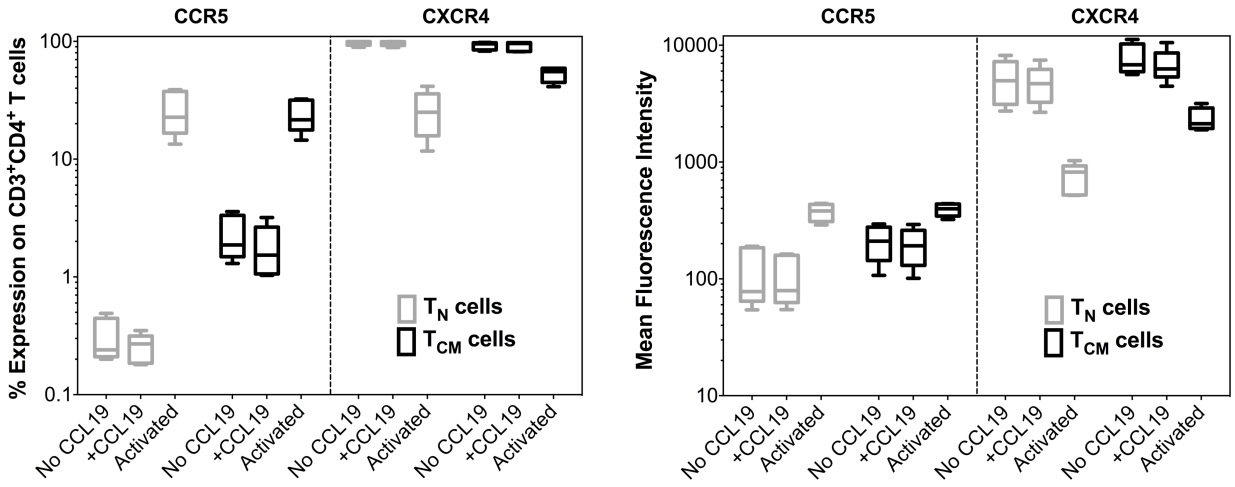


**Figure 7: CCL19 treatment does not alter the expression of HIV-1 co-receptor expression on primary CD4+ $T_N$ or $T_{CM}$ cells.** (**A**) Percent expression of CCR5 or CXCR4 on $T_N$ or $T_{CM}$ cells 2 days following treatment with CCL19 or anti-CD3/CD28 antibodies by antibody staining and flow cytometry. Untreated cells were used as a control. (**B**) CCR5 and CXCR4 surface density as assessed by mean fluorescence intensity under the same conditions as described above. No significant differences in the number of cells expressing CCR5 or CXCR4 or density of expression were noted between control cells or cells treated with CCL19 (statistics not shown; N=5).

Next, we determined whether CCL19 or direct HIV-1 infection induced T cell activation or cellular proliferation of the purified $T_N$ or $T_{CM}$ cells (Fig. 8). We found that CCL19 treatment did not up-regulate surface expression of the T cell activation markers CD25, CD69, or HLA-DR (Fig. 8A). However, a slight increase in CD25 expression was observed in all cells after 7 days of culture, which could not be attributed to HIV-1 infection (Fig. 8A). Purified rCD4 $T_N$ and $T_{CM}$ cells have been shown to express only very low levels of the intracellular proliferation marker, Ki-67, when they are freshly isolated from the blood[317]. Here, we measured cellular proliferation,

both by quantification of total cell number in culture (Fig. 8B) and by intracellular staining for Ki-67 (Fig. 8C). Importantly, we show that both CCL19 treatment and HIV-1 infection do not increase cellular proliferation throughout our experiments (Fig. 8B, 8C). It is also important to note that CCL19 treatment and HIV-1 infection did not induce cell death of either cell type (Fig. 8D).

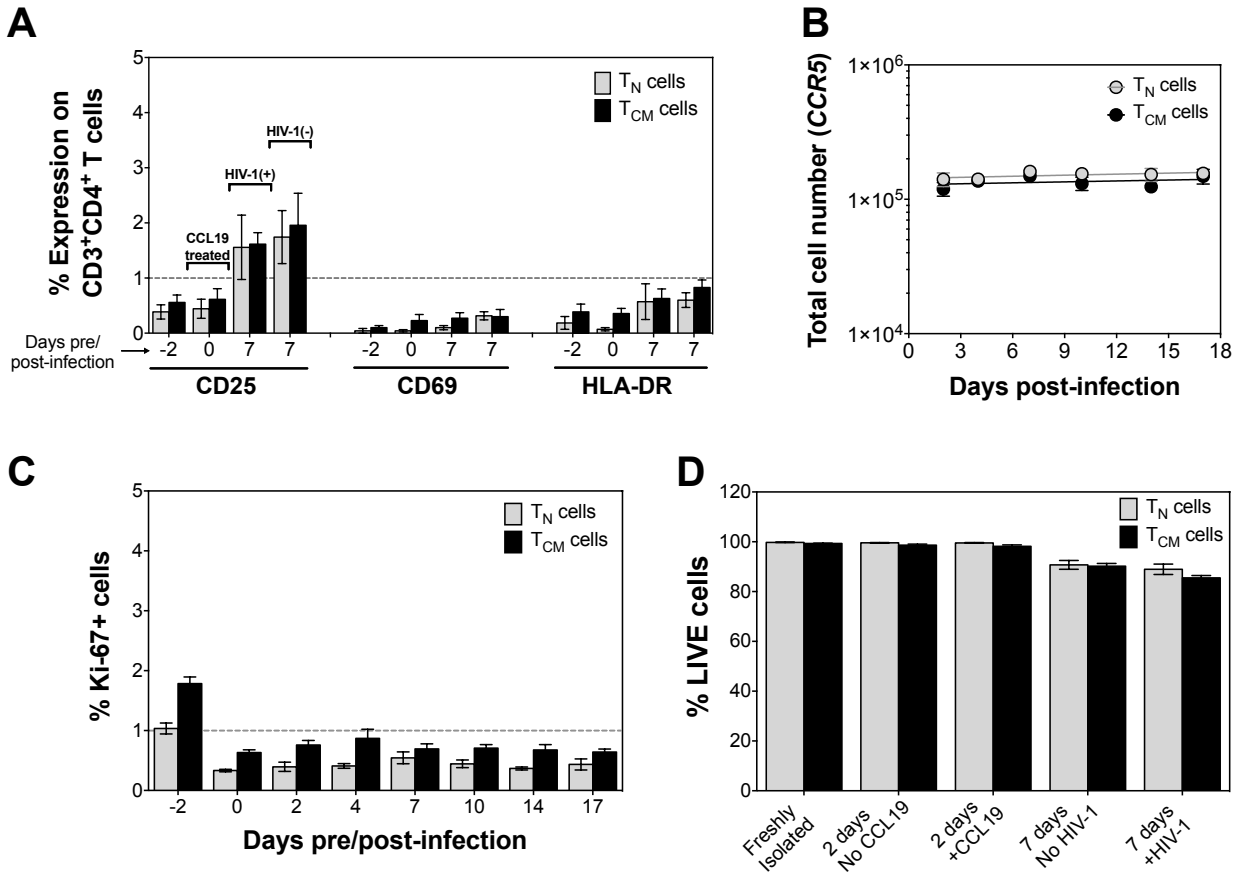


**Figure 8: T cell activation, proliferation, and cell viability of purified CD4+ $T_N$ and $T_{CM}$ cells.** (**A**) T cell activation markers CD25, CD69, and HLA-DR were assessed by antibody staining and flow cytometry on $T_N$ and $T_{CM}$ cells following purification (day -2), CCL19-treatment (day 0), HIV-$1_{LAI}$ infection (day 7, left), and no infection (day 7, right). Data are presented as the mean ± SEM (N=7). (**B**) Cellular proliferation of unstimulated cells was measured by qPCR of the *CCR5* gene throughout the time course of the experiment. Data are presented as the mean ± SEM (N=6). (**C**) Cellular proliferation was also measured by intracellular staining and flow cytometry for the proliferation marker, Ki-67. Data are presented as the mean ± SEM (N=2 performed in duplicate). (**D**) Cell viability of freshly isolated $T_N$ and $T_{CM}$ cells, after CCL19 treatment for 2 days, no CCL19 treatment for 2 days, HIV-1 infection after 7 days, and no infection after 7 days was measured by LIVE/DEAD staining and flow cytometry. Data are presented as the mean ± SEM (N=3 performed in duplicate).

### 4.2.2 Genomic distribution of HIV-1 integration sites in CD4+ $T_N$ and $T_{CM}$ cells

We next compared the genomic distribution of HIV-1 integration sites in infected $T_N$ and $T_{CM}$ cells, and, as a control, compared these values to those obtained using total CD4+ T cells that were broadly stimulated by treatment with PHA. A total of 729, 2,260 and 133,697 unique integration sites were mapped in the $T_N$, $T_{CM}$, and PHA-activated cells, respectively, with regard to several genomic annotations including RefSeq genes, transcriptional start sites (TSSs), CpG islands, and gene density (Table 3). The statistical relevance of the observed frequencies versus a matched random control (MRC) dataset was determined by Fisher's exact test for RefSeq genes, TSSs, and CpG islands, and by a Wilcoxon rank-sum test for gene density (p values provided in Supplementary Table. 1). Distributions relative to CpG islands and TSSs were calculated by counting sites that fell within a 5 kb window (+/− 2.5 kb) of these features, while gene density was calculated by counting the number of RefSeq genes falling within a 1 Mb window (+/− 500 kb) of each integration site, and then averaging this value for the entire dataset. Our MRC dataset revealed that 44.7% of human DNA comprised RefSeq genes.

**Table 3:** HIV-1 integration site preference in PHA-activated, $T_N$ and $T_{CM}$ CD4+ T cells.

| Sample | Unique integration sites | Within Refseq genes (%) | Within 5kb (± 2.5kb) of a transcriptional start site (%) | Within 5kb (± 2.5kb) of a CpG island (%) | Average gene density within 1Mb (±0.5Mb) of integration sites[a] |
|---|---|---|---|---|---|
| **Activated CD4+ T cells** | 133,697 | 114,110 (85.3)[c] | 6,242 (4.7)[c] | 7,052 (5.3)[c] | 20.3[c] |
| **CD4+ $T_N$ cells** | 729 | 613 (84.1)[c] | 30 (4.1%)[c] | 33 (4.5)[c] | 18.4[c] |
| **CD4+ $T_{CM}$ cells** | 2,620 | 2,291 (87.4)[c] | 109 (4.2%)[c] | 112 (4.3)[c] | 19.0[c] |
| **MRC[b]** | 50,000 | 22,328 (44.7) | 2,010 (4.0) | 2,100 (4.2) | 8.7 |

[a]Based on complete gene
[b]Matched random control of 50,000 computer-generated integration sites in proximity to Mse1/BglII restriction sites in hg19
[c]P values are provided in Supplementary Table. 1

As expected[623], HIV-1 targeted RefSeq genes significantly more frequently than random sequences ($T_N$, 84.1%; $T_{CM}$, 87.4%; PHA-stimulated, 85.3%; p values ranging from $3.5 \times 10^{-106}$ to $<2.2 \times 10^{-308}$). By contrast, the frequency of gene targeting across the different infection conditions was largely similar, with only minor differences noted for the $T_{CM}$ versus PHA-stimulated and $T_N$ versus $T_{CM}$ comparisons (Supplementary Table 1 in Appendix A). Integration into gene dense regions of chromosomes was similarly highly significant as compared to random, whereas the differences between infected cell datasets were largely similar (p values from 0.0002 to 0.05). As expected[624], HIV-1 integration sites nearby CpG islands or TSSs in CD4+ $T_N$ and $T_{CM}$ cells were similar to the MRC value, although small but significant differences were noted between the MRC and PHA-activated cells (Table 4; Supplementary Table 1).

### 4.2.3 CCL19-mediated HIV-1 infection of CD4+ $T_N$ and $T_{CM}$ cells is not due to an increase in filamentous actin (F-actin) density

The actin cytoskeleton is known to be a key regulator in many early HIV-1 processes including viral entry, reverse transcription, intracellular trafficking, and integration, as described in section 1.4.3.3. It has been shown that differences in actin dynamics and actin density play a major role in the differential susceptibility of $T_N$ and memory cells to HIV-1[625, 626]. Memory $CD4^+$ T cells express a higher density of cortical actin than do $T_N$ cells and also have increased actin dynamics[625, 626]. Infection of rCD4 T cells via spinoculation or CCL19 pre-treatment have both been shown to enhance HIV-1 infection by triggering cofilin dephosphorylation, actin polymerization, and enhance actin dynamics[456, 469]. Binding of HIV-1 gp120 to CXCR4 has also been shown to enhance early stages of infection in rCD4 T cells by activating cofilin, which also

results in actin polymerization and enhanced actin dynamics[454, 455, 458]. This mechanism helps explain why $T_N$ cells are more susceptible to X4-tropic strains of HIV-1 but are still less susceptible than memory cells despite having higher levels of CXCR4. Our data are consistent with this finding in which we show greater than three-fold more HIV-1 DNA in $T_N$ cells infected with HIV-$1_{LAI}$ versus HIV-$1_{BaL}$ (Figure 6B), and a 15.5 decrease in HIV-1 DNA in $T_N$ cells

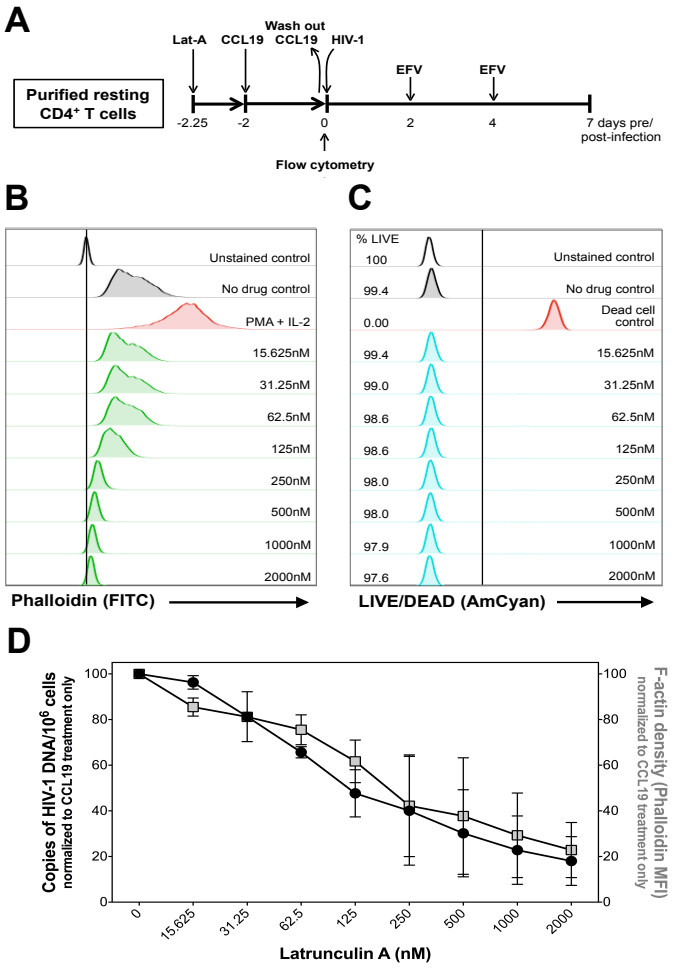


**Figure 9: Inhibition of F-actin polymerization blocks HIV-1 infection of total rCD4 T cells in a dose-dependent manner.** (**A**) Schematic representation of the experimental approach. (**B**) Cells were treated with different concentrations of lat-A for 6 h, followed by treatment with CCL19 for an additional 2 days. F-actin was stained with phalloidin and measured by flow cytometry. Cells stimulated with PMA + IL-2 were used as a positive control. Unstained cells were used as a negative control. (**C**) Following the same experimental conditions as in (**B**), cell viability was assessed by flow cytometry using LIVE/DEAD staining. Untreated cells were used as a negative control and cells heated at 56°C for 1 h prior to staining were used as a dead cell control. (**D**) F-actin density and HIV-1 infection of rCD4 T cells are plotted. Following the experimental approach shown in (**A**), HIV-1 infection was measured 7 days post-infection by quantification of total intracellular HIV-1 DNA, normalized to cell number. HIV-1 DNA and F-actin density were normalized to treatment with CCL19 only. Data shown for (**B-D**) are representative of 2 independent experiments and error bars represent standard deviations.

versus $T_{CM}$ cells infected with HIV-$1_{LAI}$ (Figure 6B, 6C).

To validate the role of F-actin density in HIV-1 infection, we first isolated total rCD4 T cells and exposed them to different concentrations of latrunculin A (lat-A), a natural product that prevents F-actin assembly, for 6 h prior to the addition of CCL19 (Fig. 9A). Forty-eight hours later, cell viability (Fig. 9B) and F-actin density (Fig. 9C) were measured by live/dead and phalloidin staining, respectively, and flow cytometry. After the 48 h treatment, cells were infected with HIV-$1_{LAI}$ and cultured for seven days (Fig. 9A). HIV-$1_{LAI}$ infection was then assessed by quantification of total HIV-1 DNA (Fig. 9D). Lat-A decreased F-actin density in a dose-dependent manner as determined by phalloidin staining intensity (Fig. 9B), but did not impact cell viability (Fig. 9C). Importantly, the decrease in F-actin density correlated with a decrease in the ability of HIV-$1_{LAI}$ to infect the rCD4 T cells (Fig. 9D).

In light of these findings, we next used confocal microscopy (Fig. 10A, 10B) and flow cytometry (Fig. 10C) to evaluate whether exposure of $T_N$ and $T_{CM}$ cells to CCL19 increased F-actin density. As described by Permanyer *et al.*, we observed a higher baseline of F-actin density in $T_{CM}$ cells compared to $T_N$ cells (Fig. 10B, 10C)[625]. However, we found no evidence that CCL19 increased F-actin density in either T cell subset after incubation with the chemokine for 2 days (Fig. 10B, 10C). It should be noted that Cameron *et al.* only observed a rapid increase in F-actin density within a few minutes of exposure to CCL19, but after 30 min no significant differences were noted[456]. Collectively, these data suggest that the very transient CCL19-mediated increase in F-actin density, reported previously, cannot explain the increased permissiveness of rCD4 T cells to HIV-1 infection 48 h post-chemokine exposure.

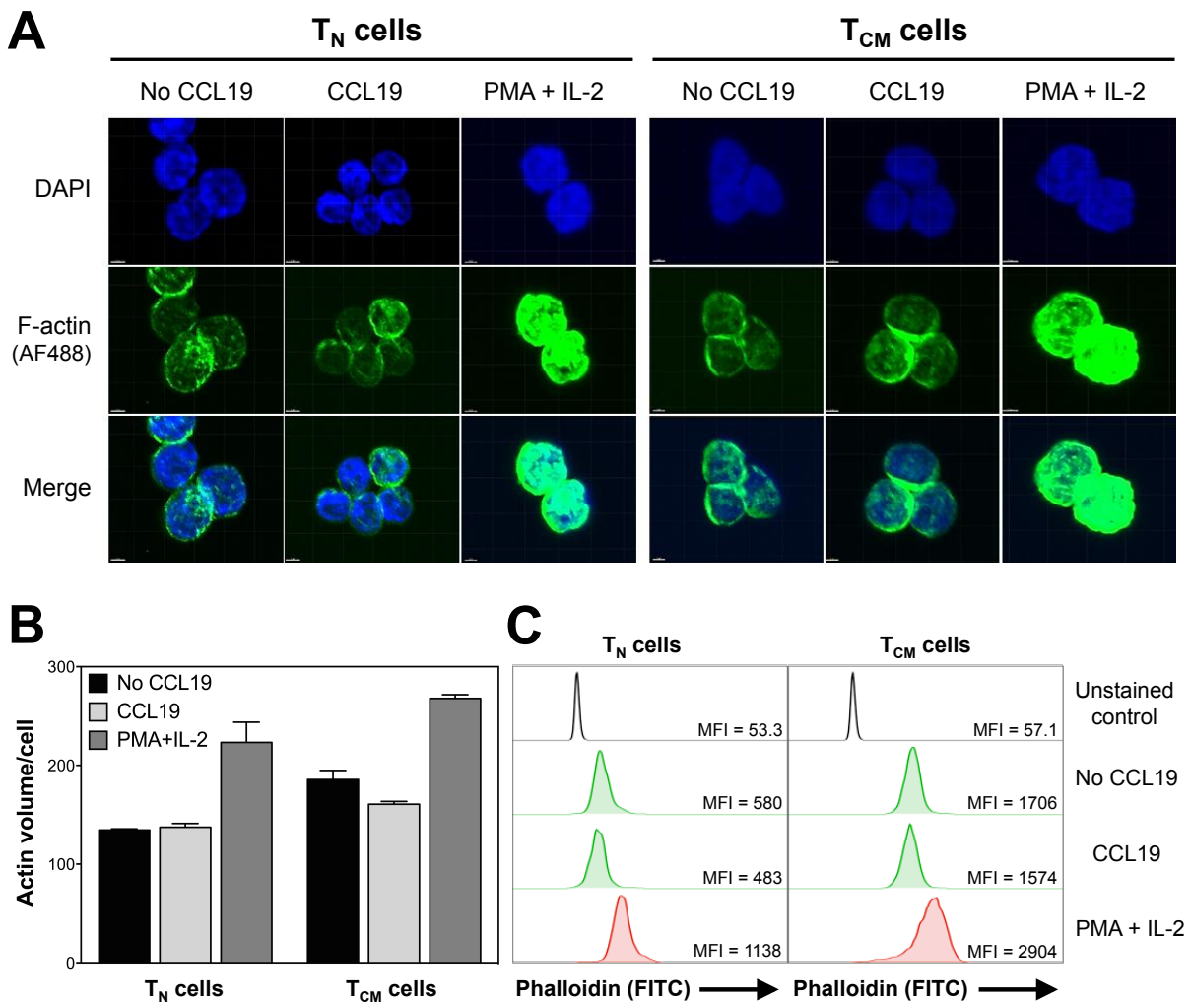


**Figure 10: CCL19 does not have an effect on F-actin density in $T_N$ or $T_{CM}$ cells.** (**A**) Representative confocal microscopy images of $T_N$ and $T_{CM}$ cells in the absence or presence of CCL19 or PMA+IL-2 for two days. Phalloidin and DAPI were used to stain F-actin and nuclei, respectively. (**B**) Total F-actin volume was measured in the $T_N$ and $T_{CM}$ cells imaged by confocal microscopy in the absence or presence of CCL19 or PMA+IL-2. Quantification was performed using Imaris software. (**C**) Flow cytometric analysis of F-actin density, measured by phalloidin staining, in $T_N$ or $T_{CM}$ cells under the same conditions as described above. Representative data from 3 independent experiments. MFI = mean fluorescence intensity.

### 4.2.4 CCL19 does not alter intracellular dNTP levels in CD4+ $T_N$ or $T_{CM}$ cells

The cellular restriction factor SAMHD1 has been shown to inhibit HIV-1 infection in rCD4 T cells, as described in section 1.4.3.3. SAMHD1 is highly expressed in resting $T_N$ and memory cell subsets. Blockade or degradation of SAMHD1 has been shown to greatly enhance the

susceptibility of rCD4 T cells to HIV-1 infection, especially in $T_N$ cells, without inducing T cell activation[344, 461]. These findings are consistent with earlier studies showing that addition of dNTPs to $T_N$ or memory cells significantly enhanced reverse transcription and integration[627, 628]. Differences in cellular restriction factors, such as SAMHD1, could also help explain the differential susceptibility of $T_N$ and $T_{CM}$ cells to HIV-1 infection.

**Table 4:** dNTP concentrations in purified $T_N$ and $T_{CM}$ cells following treatment with either CCL19 or anti-CD3/CD28.

| Cell type and treatment | Nucleotide Concentration (fMol/$10^6$ cells)[ρ] | | | |
|---|---|---|---|---|
| | dATP | dGTP | dCTP | dTTP |
| **$T_N$ cells** | 8.70 (<4 – 10.7) | 32.0 (14.6 - 44.5) | 27.7 (<4 – 43.1) | 5.90 (<4 – 7.60) |
| **$T_N$ cells + CCL19** | 4.70 (<4 – 6.10) | 28.2 (17.5 – 29.4) | 14.7 (<4 – 30.6) | 4.70 (<4 – 7.30) |
| **$T_N$ cells + anti-CD3/CD28** | 379 (112 - 513) | 484 (404 - 622) | 88.6 (62.7 - 111) | 649 (244 - 735) |
| **$T_{CM}$ cells** | 3.70 (<4 – 5.8) | 30.0 (19.5 – 77.1) | 11.5 (5.75 – 21.0) | 4.40 (<4 – 6.90) |
| **$T_{CM}$ cells + CCL19** | 4.10 (<4 – 4.90) | 24.6 (11.6 – 61.6) | 10.0 (4.80 – 17.3) | 4.90 (<4 – 12.3) |
| **$T_{CM}$ cells + anti-CD3/CD28** | 285 (57.2 - 294) | 232 (217 - 345) | 41.0 (31.9 – 61.8) | 164 (106 - 188) |

[ρ] Nucleotide concentrations were measured following a two-day treatment with either CCL19 or anti-CD3/CD28 and were compared to untreated control cells. Data are presented as the median (range) of three independent experiments. < 4 indicates below the limit of detection based on the number of cells used for quantification.

Given these findings, we asked whether CCL19 enhanced HIV-1 permissivity of $T_N$ and $T_{CM}$ cells by increasing intracellular dNTP levels, which in turn would facilitate HIV-1 reverse transcription and viral infection. Nucleotide levels in the $T_N$ and $T_{CM}$ cells were much lower than in anti-CD3/CD28-activated cells (Table 4). However, treatment of $T_N$ or $T_{CM}$ cells with CCL19 did not increase the intracellular dNTP levels compared to the untreated controls (Table 4), which suggests that CCL19 does not enhance HIV-1 infection of $T_N$ and $T_{CM}$ cells by increasing the nucleotide pool concentration.

### 4.2.5 Latency reversal from CD4+ $T_N$ and $T_{CM}$ Cells infected with HIV-$1_{LAI}$

We next quantified total virus production (i.e. extracellular virion-associated RNA (vRNA) in the culture supernatant) from latently-infected $T_N$ and $T_{CM}$ cells using an ultrasensitive assay capable of single copy detection of HIV-1 RNA[160, 629] before and after exposure to the LRAs: (i) anti-CD3/CD28 antibodies; (ii) PMA + PHA; (iii) prostratin; or (iv) SAHA (Fig. 11A). As expected, given that the $T_{CM}$ cells contained significantly higher levels of HIV-1 DNA than did the $T_N$ cells, we saw more total virus production from $T_{CM}$ cells than from the $T_N$ cells (Fig. 11B). To account for differences in HIV-1 infection frequency between the $T_N$ and $T_{CM}$ cell subsets, as well as between different donors, we normalized extracellular vRNA production to the total HIV-1 DNA copy number per cell at each respective time point (Fig. 11C). Surprisingly, our data revealed that $T_N$ cells exposed to anti-CD3/CD28 antibodies, PMA+PHA, or prostratin yielded as much, or more, vRNA than did the $T_{CM}$ cells (Fig. 11C). For example, at day 10 the median production of extracellular HIV-$1_{LAI}$ vRNA after exposure to anti-CD3/CD28 antibodies, PMA+PHA, or prostratin was 69.7, 71.5, and 29.6 from $T_N$ cells, compared to 18.8, 57.2, and 21.2 from $T_{CM}$ cells, respectively. There was, however, significant variation between donors (Fig. 11D) For example in donors 2, 4, 5, and 6 more HIV-$1_{LAI}$ vRNA was produced from the $T_N$ cells; whereas more HIV-$1_{LAI}$ vRNA was produced from the $T_{CM}$ cells of donors 1 and 3 (Fig. 11D). As such, the differences between the two cell types were not statistically significant.

Collectively, these data suggest that donor genetic differences, in addition to the rCD4 T cell compartment, impact the establishment and reversal of HIV-1 latency. In contrast to the other LRAs, SAHA did not significantly increase vRNA production in either $T_N$ or $T_{CM}$ cells (Fig. 11B, 11C). This finding is consistent with recent studies that demonstrated the inability of

SAHA to increase extracellular HIV-1 production from rCD4 T cells isolated from infected individuals on suppressive ART[482, 512, 630].

Viral integration into the host cell genome is an essential step in HIV-1 replication, yet many reverse transcripts fail to integrate, resulting in the accumulation of viral DNA that has the potential to persist in rCD4 T cells. The stability of unintegrated forms of HIV-1 DNA in rCD4 T cells has been somewhat controversial. While some studies have shown that unintegrated forms of HIV-1 DNA are short-lived and degraded rapidly[631-634], other studies have reported that unintegrated forms of HIV-1 DNA can persist for several days or weeks in rCD4 T cells[374, 635-637]. Regardless of the longevity of unintegrated forms of HIV-1 DNA, it has been shown that upon CD4+ T cell activation, some forms of unintegrated HIV-1 DNA can become integrated, and can result in productive infection[634, 637-639]. Because anti-CD3/CD28 antibodies induce T cell activation, we next assessed the contribution of unintegrated HIV-$1_{LAI}$ DNA to extracellular vRNA production from both $T_N$ and $T_{CM}$ cells by including the integrase inhibitor, RAL, at the same time as anti-CD3/CD28 (Fig. 11E, 11F). This analysis suggested that unintegrated viral DNA did not significantly contribute to the extracellular vRNA quantified in the supernatant following reversal of latency in our model system.

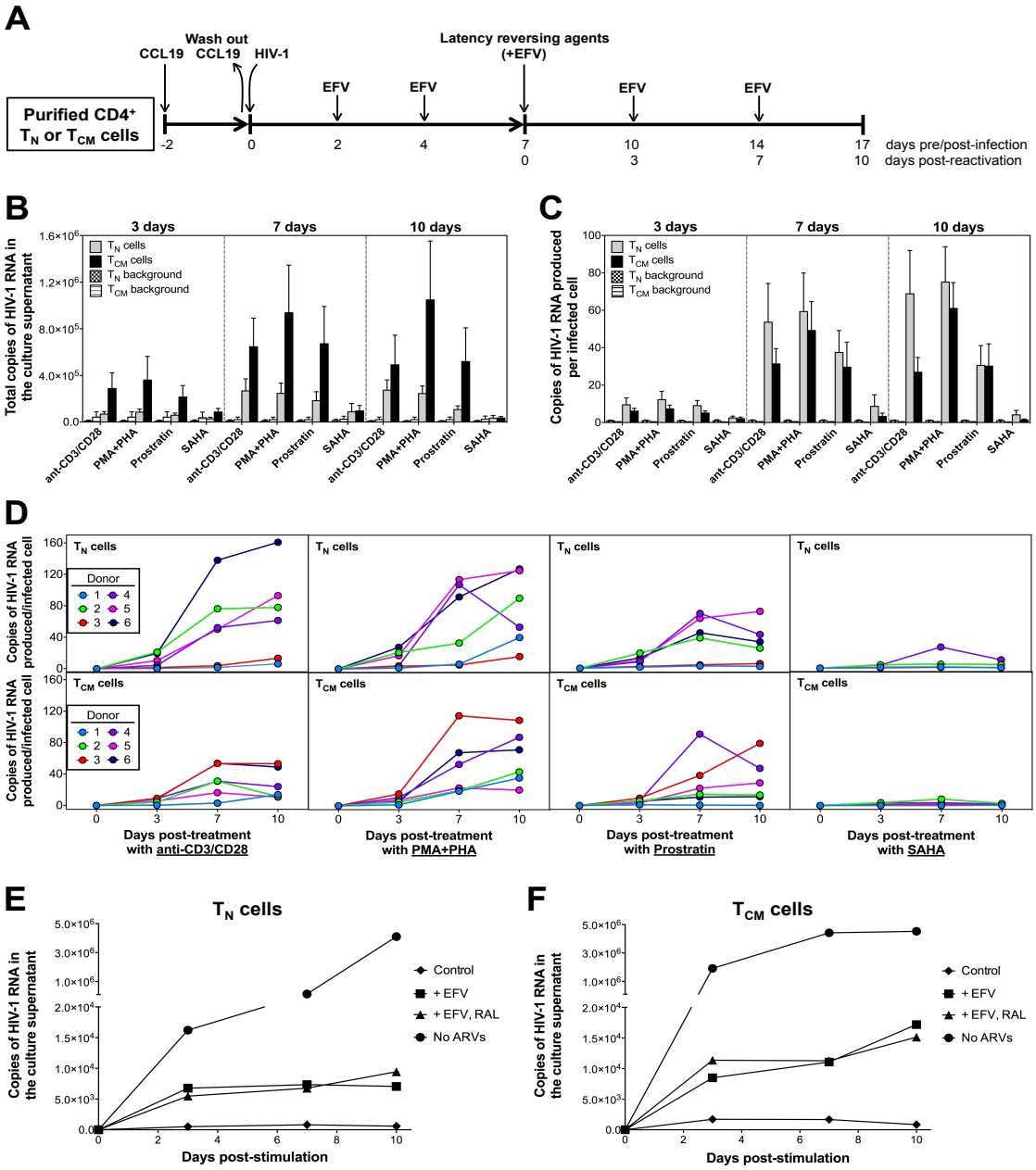


**Figure 11: Reversal of HIV-1 latency in CD4+ $T_N$ and $T_{CM}$ cells infected with HIV-$1_{LAI}$ following treatment with LRAs.** (**A**) Schematic representation of the experimental approach. (**B**) Total copies of extracellular virion-associated HIV-$1_{LAI}$ RNA produced from $T_N$ or $T_{CM}$ cell after exposure to anti-CD3/CD28 antibodies, PMA+PHA, prostratin, or SAHA. Background HIV-1 RNA from unstimulated controls at each time point are shown. Data are shown as the mean ± the SEM from 6 donors. (**C**) Copies of extracellular virion-associated HIV-$1_{LAI}$ RNA produced per infected $T_N$ or $T_{CM}$ cell after exposure to anti-CD3/CD28 antibodies, PMA+PHA, prostratin, or SAHA, normalized to the level of infection at each respective time point. Background HIV-1 RNA from unstimulated controls at each time point are shown. Data are shown as the mean ± the SEM from 6 donors. (**D**) Copies of extracellular virion-associated HIV-$1_{LAI}$ RNA produced per infected $T_N$ or $T_{CM}$ cell after exposure to anti-CD3/CD28 antibodies from 6 donors. Contribution of unintegrated HIV-$1_{LAI}$ DNA to the level of extracellular HIV-1 RNA copy number produced after exposure of infected (**E**) $T_N$ cells or (**F**) $T_{CM}$ cells to anti-CD3/CD28 antibodies was determined with or without EFV only or EFV + RAL treatment throughout the experiment. Unstimulated cells treated with EFV only were used as a control. Cells stimulated in the absence of any antiretroviral drugs were used as a positive control. Data are representative of 2 independent experiments.

### 4.2.6 Correlation between level of infection and virion production from latently infected $T_N$ and $T_{CM}$ cells

Given the differences in infection frequency (Fig. 6B, 6C) and virion production post-stimulation (Fig. 11B, 11C), we wanted to determine if there was a correlation between virion production post-stimulation and the frequency of infection between $T_N$ and $T_{CM}$ cells. Following stimulation of $T_N$ cells with anti-CD3/CD28, PMA+PHA, and prostratin, there was no correlation between virion production and level of infection at 3 (Supplementary Fig. 1A), 7 (Fig. 12A), and 10 days post-stimulation (Supplementary Fig. 1B). However, there was a strong correlation between virion production and level of infection from $T_{CM}$ cells with all three treatments at 3 (Supplementary Fig. 1C), 7 (Fig. 12B), and 10 days post-stimulation (Supplementary Fig. 1D). There was no correlation from either cell type between copies of HIV-1 RNA produced per infected cell versus frequency of infection (data not shown). These data suggest that there are different mechanisms or different mechanistic regulations promoting or suppressing virion production following latency reversal.

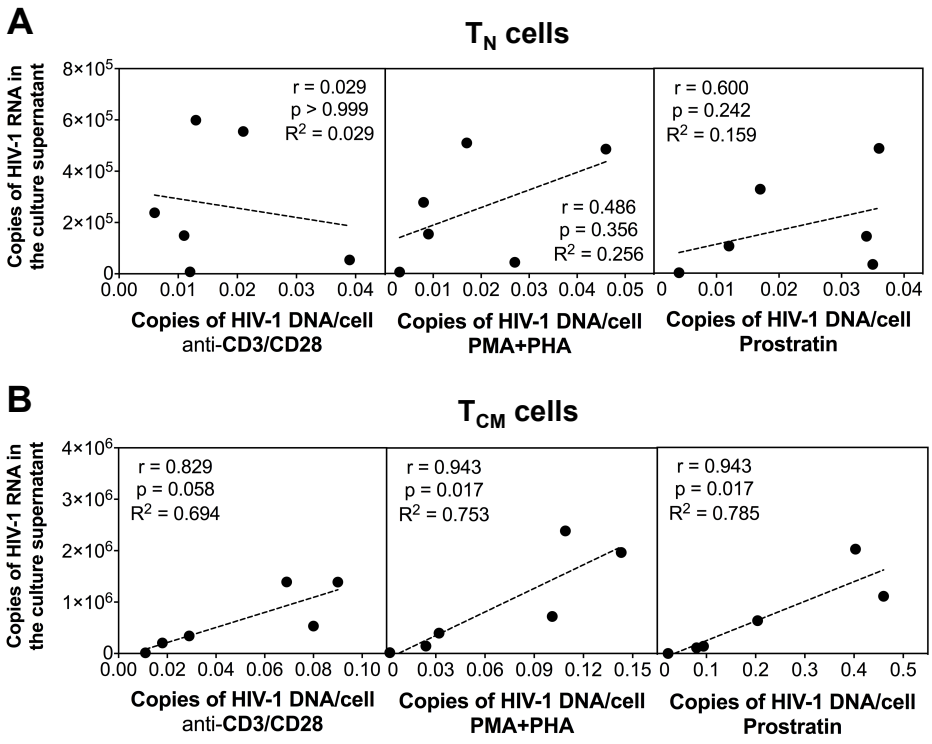


**Figure 12: Correlation analyses between level of infection and virus production seven days post-stimulation from $T_N$ and $T_{CM}$ cells latently infected with HIV-$1_{LAI}$.** Correlation analysis of $T_N$ cells (**A**) and $T_{CM}$ cells (**B**) seven days post-stimulation with anti-CD3/CD28 microbeads, PMA+PHA, and prostratin. P value determined by Spearman correlation analysis. Spearman correlation coefficients (r) are shown. Each dot represents a different donor. Dotted lines represent linear regression curve.

### 4.2.7 Latency reversal from CD4+ $T_N$ and $T_{CM}$ cells infected with HIV-$1_{BaL}$

We also evaluated extracellular HIV-$1_{BaL}$ RNA production from infected $T_N$ and $T_{CM}$ cells exposed to the same LRAs, however, we excluded SAHA given that we did not see an effect in our previous experiments (Fig. 11B). HIV-$1_{BaL}$ vRNA was produced from $T_{CM}$ cells, with no differences noted compared to HIV-$1_{LAI}$ (Fig. 13). In contrast, almost no detectable HIV-$1_{BaL}$ vRNA was produced from the $T_N$ cells (data not shown). However as noted in Figure 6C, these cells were largely refractory to infection by HIV-$1_{BaL}$.

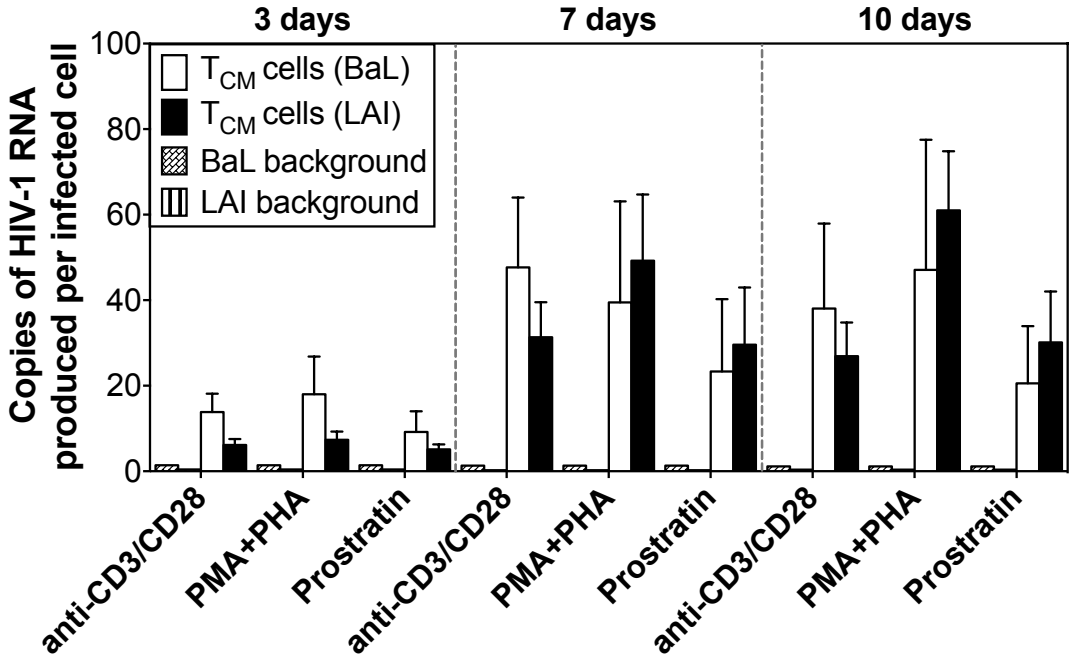


**Figure 13: Reversal of HIV-1 latency in CD4+ $T_{CM}$ cells infected with HIV-$1_{BaL}$ following treatment with LRAs.** The experimental approach was the same here as shown in Figure 11A. Copies of extracellular virion-associated HIV-$1_{BaL}$ RNA produced per infected $T_{CM}$ cell after exposure to anti-CD3/CD28 antibodies, PMA+PHA, or prostratin, normalized to the level of infection at each respective time point. Background HIV-1 RNA from unstimulated controls at each time point are shown. Data are presented as the mean ± SEM from 3 donors. For comparative purposes, data for HIV-$1_{LAI}$ RNA from infected $T_{CM}$ cells (Fig. 11C) are included.

### 4.2.8 Decay of HIV-$1_{LAI}$-infected CD4+ $T_N$ and $T_{CM}$ cells after exposure to LRAs

Lastly, we measured the decay kinetics of HIV-$1_{LAI}$-infected cells in both T cell populations after exposure to the different LRAs (Fig. 14A, 14B). The rates of decay ($t_{1/2}$) of the HIV-$1_{LAI}$-infected $T_N$ cells treated with anti-CD3/CD28, PMA+PHA, or prostratin were 4.2, 3.5, and 4.8 days, respectively (Fig. 14A), which were similar to the values calculated for the $T_{CM}$ cells (2.6, 2.5, and 5.2 days for cells treated with anti-CD3/CD28, PMA+PHA, or prostratin, respectively) (Fig. 14B). No appreciable decay was observed in either $T_N$ or $T_{CM}$ cells treated with SAHA. Of note, the anti-CD3/CD28 antibodies, PMA+PHA, and prostratin all induced cellular proliferation

(Fig. 14C) and T cell activation in both T cell subsets, as evidenced by increased expression of CD69, CD25, and HLA-DR (Figure 14C). The level of cellular activation was also similar between $T_N$ and $T_{CM}$ cells and likely does not contribute to the differences observed in virus production between these cell types.

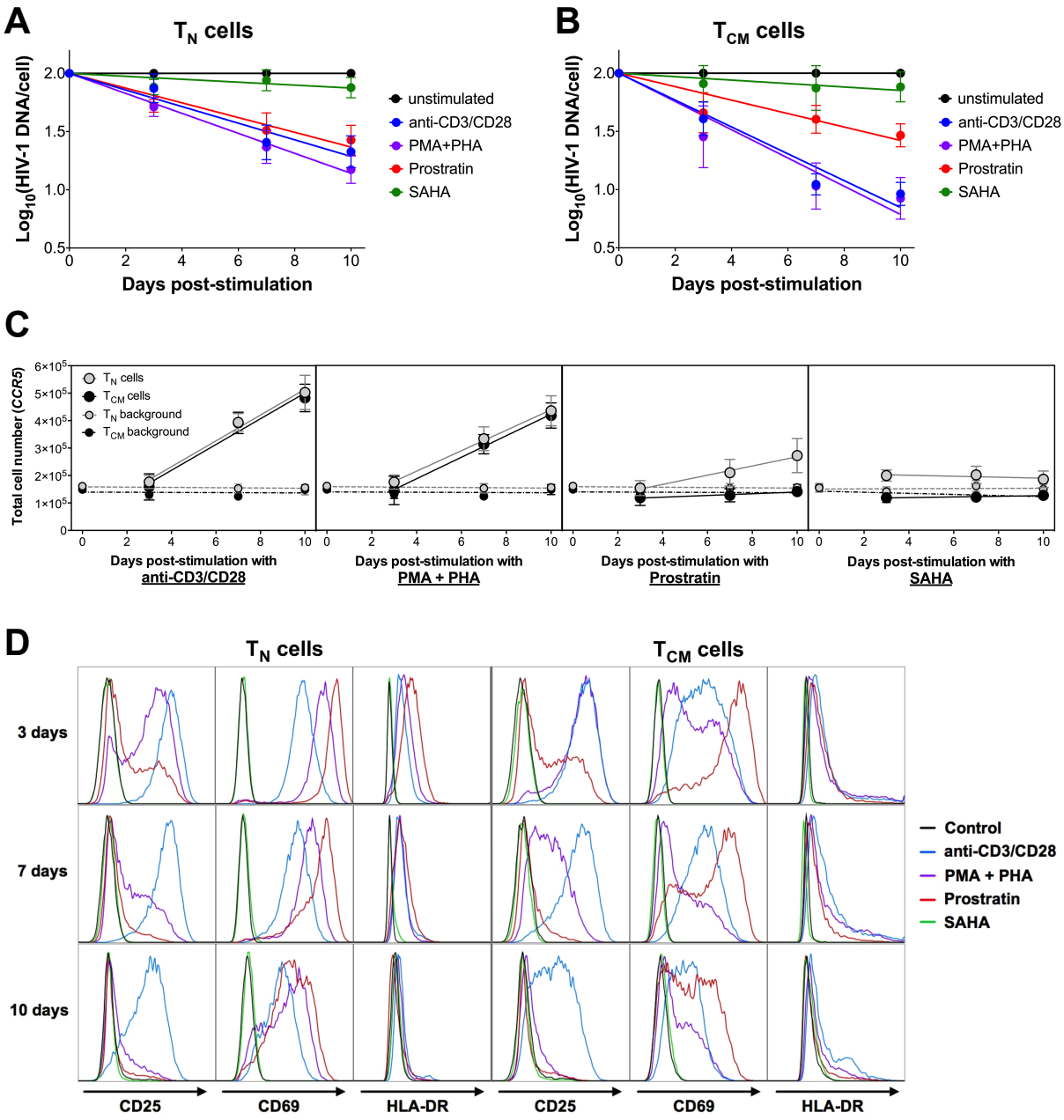


**Figure 14: Decay of HIV-$1_{LAI}$-infected cells and T cell activation post-treatment of latently infected CD4+ $T_N$ and $T_{CM}$ cells.** Decay of HIV-$1_{LAI}$-infected (**A**) $T_N$ or (**B**) $T_{CM}$ cells was measured over 10 days of treatment with anti-CD3/CD28 antibodies, PMA+PHA, prostratin, or SAHA. Total intracellular HIV-1 DNA was quantified as described in Figure 5. Data are presented as the mean ± SEM from 6 donors (except for the SAHA data, where N=4). (**C**) Cellular proliferation of $T_N$ and $T_{CM}$ cells was measured by qPCR of the *CCR5* gene following stimulation with anti-CD3/CD28 antibodies, PMA+PHA, prostratin, or SAHA. Background samples represent unstimulated CD4+ $T_N$ and $T_{CM}$ cells. Data are presented as the mean ± SEM (N = 6, except for SAHA data where N = 4). (**D**) T cell activation was measured by antibody staining and flow cytometry for surface expression of CD25, CD69 and HLA-DR on $T_N$ or $T_{CM}$ cells 3, 7, and 10 days after treatment with anti-CD3/CD28 antibodies, PMA+PHA, prostratin, or SAHA. Untreated cells were used as a negative control. Data are representative of 2 independent experiments.

## 4.3 DISCUSSION

Latently infected rCD4 T cells constitute the major reservoir of persistent HIV-1 infection, and significant reduction or elimination of this reservoir could lead to either a functional or sterilizing cure, respectively. It has been hypothesized that therapeutic approaches that reactivate latent HIV-1 infection will promote death of the infected cell by viral cytopathic effects and/or by host cell effector mechanisms[640]. This strategy is typically referred to as the "kick and kill" approach[488]. The rCD4 T cell population, however, is heterogeneous and consists of different T cell subsets, including naïve and memory cells. It is not known whether the "kick and kill" approach will be equally effective in the different T cell subsets, which differ in lifespan, proliferative capacity, antigen response time, residence throughout the body, and in their HIV-1 co-receptor, CCR5 and CXCR4, expression levels[355, 620, 621]. Therefore, mechanistic insights into the establishment and reversal of latent HIV-1 infection in different rCD4 T cell subsets could provide important clues to eradicating this persistent reservoir.

In this study, we compared the establishment and reversal of HIV-1 latency in rCD4 $T_N$ and $T_{CM}$ cells using a primary cell model of latency that utilizes direct infection of highly purified cells. Prior studies demonstrated that HIV-1 latency could be established *in vitro* in rCD4 T cells pre-treated with chemokines that bind to the CCR6, CCR7 or CXCR3 receptors[456]. From an important biological perspective, the concentration of CCL19 (the chemokine ligand for CCR7 that was used in this study) significantly increases during acute HIV-1 infection, in which the latent reservoir is established, and correlates with disease progression[641-644]. Furthermore, pre-treatment of $T_N$ or $T_{CM}$ cells with CCL19 does not induce T cell activation or proliferation (Fig. 8). As such, the integrity of the purified T cell subsets is largely preserved in the experiment, which was an important prerequisite for the objectives of this study.

We show that CCL19 pre-treatment of $T_N$ and $T_{CM}$ cells significantly increased the capacity for X4-tropic HIV-1 to infect these cells (Fig. 6). However, as reported in other in vitro systems[340, 344, 627], the levels of HIV-1 infection in the $T_{CM}$ cells, as assessed by quantitation of total viral DNA, were higher compared to the $T_N$ cells. In contrast, CCL19 only increased R5-tropic virus infection of $T_{CM}$ cells but not $T_N$ cells, a finding which may be attributable to the extremely low levels of CCR5 expression on $T_N$ cells isolated from healthy donors (Fig. 7). However, several studies have demonstrated that R5-tropic virus can be isolated from CD4+ $T_N$ cells from HIV-infected individuals[309, 311, 645, 646]. There are two possible explanations that could account for the discrepancies between in vitro studies and the in vivo observations: (i) HIV-1 infection systematically upregulates CCR5 expression on $T_N$ cells, thus making them more susceptible to infection; and/or (ii) R5-tropic virus may be more efficiently transferred to $T_N$ cells by plasmacytoid dendritic cells. Previous studies have shown that CCR5 expression is systemically upregulated following HIV-1 infection on CD4+ T cells as well as on purified $T_N$ cells isolated from HIV-1-infected individuals[647-651]. It has also been shown that monocyte-derived dendritic cells (MDDCs) and primary isolated plasmacytoid dendritic cells (pDCs) are capable of transferring R5-tropic HIV-1 to lymphocytes[652-654]. Both explanations are possible, however, further studies are needed to elucidate the mechanism(s) by which $T_N$ cells become infected in vivo.

We also attempted to address the mechanism by which CCL19 increases the ability of HIV-1 to infect $T_N$ and $T_{CM}$ cells. In this regard, Cameron et al.[456] reported that ligation of the CCR6, CCR7, and CXCR3 receptors led to changes in cortical actin, allowing for rapid migration of the pre-integration complex to the nucleus and efficient nuclear localization and integration. While our data show a critical role for F-actin density in the ability of HIV-1 to

infect rCD4 T cells (Fig. 9D), and suggest that $T_N$ cells may be less susceptible to HIV-1infection than $T_{CM}$ cells due to a lower F-actin density as reported previously[625, 626], our comprehensive imaging and flow cytometry data do not support a role for CCL19 in increasing F-actin density in either cell type (Fig. 10).

SAMHD1 has also been identified as an effective restriction factor to HIV-1 infection in rCD4 T cells by enzymatically decreasing cellular dNTP pools and impeding HIV-1 reverse transcription[355, 460, 462, 463]. Blockade or degradation of SAMHD1 has been shown to greatly enhance the susceptibility of rCD4 T cells to HIV-1 infection, especially in $T_N$ cells[344, 461]. These findings are consistent with earlier studies showing that addition of dNTPs to $T_N$ or memory cells significantly enhanced reverse transcription and integration[627, 628]. In this study, we found that CCL19 does not alter intracellular dNTP concentrations (Table 3), and that HIV-1 can infect both $T_N$ and $T_{CM}$ cells despite having low nucleotide concentrations (Fig. 6). Collectively, these data suggest that CCL19 pre-treatment could potentially impact an as yet undocumented restriction factor in rCD4 T cells.

An unexpected finding from this study was that $T_N$ cells exposed to LRAs produced more extracellular virion-associated RNA per infected cell compared to the $T_{CM}$ population (Fig. 6). This observation was found to be independent of the LRA used. These data suggest one of two things: (i) either more latently infected $T_N$ cells produce virus than $T_{CM}$ cells, or (ii) more virus is produced per infected $T_N$ cell versus $T_{CM}$ cell. We observed no major differences in the genomic distribution of HIV-1 integration sites between the two T cell subsets (Table 4). Given these differences in virus production between $T_N$ and $T_{CM}$ cells, we also evaluated whether HIV-1 reactivation resulted in death of the infected cell. We found that the rates of decay of the HIV-1-infected cells in the $T_N$ and $T_{CM}$ populations treated with anti-CD3/CD28 antibodies,

PMA+PHA, or prostratin were largely equivalent (Fig. 14A, 14B). Using a different in vitro primary cell model of latency, Shan *et al.* reported that if the LRA induced T cell activation, reactivation of latent HIV-1 resulted in death of the infected cell[655]. Our data support this finding. However, the authors also reported that following administration of SAHA, which does not induce T cell activation, the HIV-infected rCD4 T cell population survived, even in the presence of autologous cytolytic T lymphocytes[655]. In other words, SAHA facilitated the "kick" but not the "kill". In contrast, we observed that SAHA promoted neither reactivation of latent HIV-1 nor death of the infected cell.

In conclusion, this study highlights the differences of the establishment and reversal of HIV-1 latency in $T_N$ and $T_{CM}$ cells. Importantly, the data reveal that despite low infection frequency, $T_N$ cells produce more extracellular virion-associated RNA per infected cell than $T_{CM}$ cells. This suggests that $T_N$ cells may be an important reservoir of latent HIV-1 infection, and should not be ignored simply because the frequency of infection of these cells is lower than in the memory T cell subsets in infected individuals on ART. Importantly, we have presented a novel approach to study HIV-1 latency in a primary cell model, focusing specifically on $CD4^+$ $T_N$ and $T_{CM}$ cell subsets that can further be used to understand the establishment, maintenance, and reversal of latency in both subsets.

# 5.0 NAÏVE CD4+ T CELLS HARBOR A LARGE INDUCIBLE RESERVOIR OF LATENT HIV-1 IN INFECTED INDIVIDUALS ON LONG-TERM SUPPRESSIVE ART

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Author contributions: J.M.Z., J.W.M., and N.S.C. conceived of the study and performed data analysis and interpretations. M.S. isolated PBMCs from leukophereses products from HIV-1-infected individuals. J.M.Z. isolated all other CD4+ T cell subsets from the PBMCs. J.M.Z. performed all experiments and statistical analyses. D.M. enrolled HIV-1-infected individuals in the study and provided leukophereses products.

## 5.1 INTRODUCTION

It has long been known that HIV-1 latency is primarily established in rCD4 T cells[155-157]. A more detailed analysis of the composition of this reservoir of latently infected cells in individuals on suppressive ART, based on quantification of HIV-1 DNA and the frequency of each cell type in the peripheral blood, revealed that $T_{CM}$ cells were the major contributing cell type to the latent reservoir, followed by $T_{TM}$ cells and $T_{EM}$ cells, while $T_N$ cells contributed only minorly[317]. Many studies since then have further characterized the CD4+ T cell subset distribution of the HIV-1 DNA reservoir, consistently finding that either $T_{CM}$ and/or $T_{TM}$ cells were the major contributing cell types to the latent reservoir, while $T_N$ and $T_{EM}$ cells showed slightly more variation[316, 340, 360, 600]. In light of these findings, the vast majority of in vitro and ex vivo latency reversal studies have focused on resting memory CD4+ T cells or memory T cell subsets, and have largely overlooked the $T_N$ cell subset.

In this study, we wanted to extend our findings from chapter 4.0, using an in vitro primary cell model of HIV-1 latency, to ex vivo cells from HIV-1-infected individuals. Using our in vitro primary cell model, we found that $T_N$ cells contributed significantly to virus production following latency reversal, despite having lower levels of HIV-1 DNA. While previous studies measuring the contribution of different T cell subsets to the HIV-1 latent reservoir have largely neglected $T_N$ cells altogether[342], or analyzed their significance on a population level[317, 360, 600], here, using ultrasensitive quantitative analyses, we were able to determine the significance of the $T_N$ cell population, in comparison to the $T_{CM}$ cell population, on a per cell basis.

## 5.2 RESULTS

### 5.2.1 Study participant characteristics and baseline measurements

This study enrolled 7 long-term suppressed HIV-1-infected individuals from the Pitt AIDS Center for Treatment in an IRB-approved study. All study participants gave informed, written consent at the time of enrollment. The participant characteristics are shown in Table 5. Plasma HIV-1 RNA was suppressed by ART for an average of 9.5 years in the study participants. The mean age of the participants was 52 years (range 36-62 years); of which 57% were female, and 71% were African American, with the remaining 29% being Caucasian.

**Table 5:** Baseline characteristics of study participants.

| | Sex | Age (years) | Race | Current CD4$^{\tau}$ | Pre-ART viral load | Years of suppression$^{\rho}$ | Current ART regiment |
|---|---|---|---|---|---|---|---|
| **Donor 1** | Female | 36 | African American | 809 | 802,000 | 10.2 | FTC/RPV/TDF |
| **Donor 2** | Female | 57 | African American | 380 | 143,000 | 5.2 | FTC/TDF/r/ATZ |
| **Donor 3** | Male | 57 | Caucasian | 656 | 520,000 | 12 | EFV/FTC/TDF |
| **Donor 4** | Male | 51 | Caucasian | 603 | Unknown | 7.3 | EVG/c/FTC/TAF |
| **Donor 5** | Male | 46 | African American | 714 | 41,952 | 6.0 | 3TC/ABC/EFV |
| **Donor 6** | Female | 58 | African American | 1426 | 366,200 | 15 | FTC/RPV/TDF |
| **Donor 7** | Female | 62 | African American | 1033 | Unknown | 10.7 | DTG/FTC/TDF |
| **Mean** | - | 52 | - | 803 | - | 9.5 | - |

$^{\tau}$ Current CD4$^{+}$ T cell count given as cells/μL
$^{\rho}$ Years of suppression without ART failure

HIV-1 DNA was quantified in total CD4+ T cells (tCD4s) as well as activated (aCD4s) and rCD4s, $T_N$, $T_{CM}$ and $T_{TM}$+$T_{EM}$ cells (Fig. 15A). The $T_{TM}$+$T_{EM}$ cell population was not further subdivided for DNA analysis. There were slightly higher levels of HIV-1 DNA in the rCD4 T cell compartment versus the activated compartment; however, this difference was not significant. Consistent with previous studies[313, 340, 360, 600], we found significantly higher levels of HIV-1 DNA in $T_{CM}$ cells versus $T_N$ cells in all 7 donors ($p = 0.0175$) with a 4.2 median fold increase, ranging from 1.2 - 14.7. Interestingly, we also found higher levels of HIV-1 DNA in the $T_{TM}$+$T_{EM}$ cells compared to the $T_{CM}$ cells in all 7 donors, with a 1.5 median fold increase, ranging from 1.1 – 3.0. This increase in HIV-1 DNA was not significantly higher than that seen in $T_{CM}$ cells, however, it was significantly higher than that in $T_N$ cells ($p = 0.0006$).

During HIV-1 infection, the composition and the distribution of T cell subsets change compared to that seen in uninfected individuals, somewhat complicating the analysis of significance for each T cell subset. In healthy, HIV-1-uninfected individuals, the primary rCD4 T cell type in the peripheral blood is $T_N$ cells, accounting for nearly 40-50% of the total population, closely followed by $T_{CM}$ cells, accounting for approximately 30% of the total population (Fig. 15B)[351]. In HIV-1-infected individuals, however, the frequency of these two cell types in the peripheral blood becomes reversed, while the frequency of $T_{TM}$, $T_{EM}$, and $T_{TD}$ cells remains largely the same (Fig. 15B)[317, 351]. When we measured the contribution of HIV-1 DNA in the reservoir of rCD4 T cells, taking into consideration the frequency of HIV-1 DNA in each subset compared to the total amount of HIV-1 DNA in the reservoir, we found that $T_{TM}$+$T_{EM}$ cells contributed significantly higher to the DNA reservoir than $T_{CM}$ cells, which in turn was significantly higher than in $T_N$ cells (Fig. 15C). However, when we also took into consideration the frequency of each cell subset in the peripheral blood to calculate the percent contribution to

the HIV-1 reservoir in rCD4 T cells, we found that $T_{CM}$ cells were the major contributing cell type (Fig. 15D).

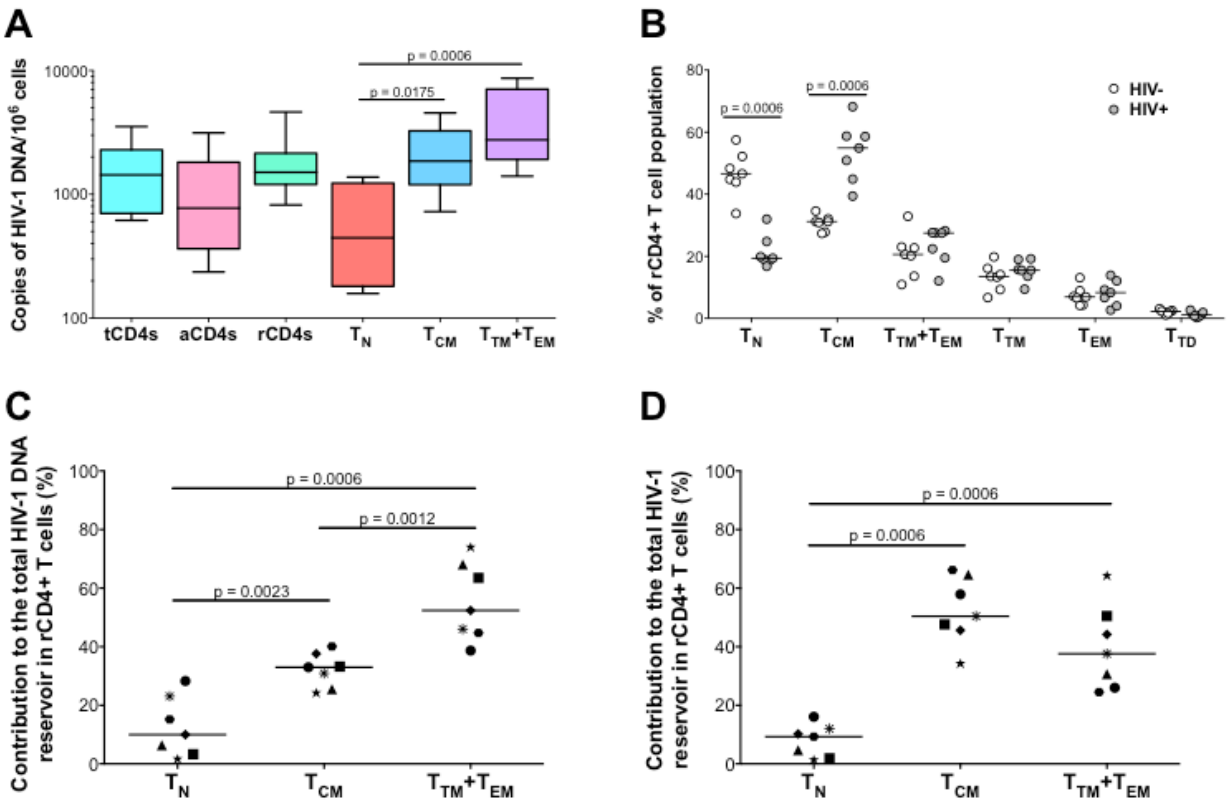


**Figure 15: Quantification of HIV-1 DNA in freshly isolated CD4+ T cell subsets from long-term suppressed HIV-1-infected individuals. (A)** HIV-1 DNA was quantified in various CD4+ T cell subsets following purification. Total HIV-1 DNA was normalized to cell number assayed by quantification of the *CCR5* gene. (**B**) The frequency of rCD4 T cell subsets were measured in the peripheral blood of 7 HIV-1-infected and 7 HIV uninfected donors. (**C**) The percent contribution of HIV-1 DNA in $T_N$, $T_{CM}$, and $T_{TM}$+$T_{EM}$ cells to the total HIV-1 DNA pool among these three cell populations was calculated. (**D**) The percent contribution of $T_N$, $T_{CM}$, and $T_{TM}$+$T_{EM}$ cells to the total HIV-1 latent reservoir in rCD4 T cells was calculated. For C and D, each symbol represents a unique donor. All p values were determined using a Mann-Whitney test.

More recent studies have identified an additional CD4+ T cell subset that contributes to the HIV-1 latent reservoir, and under conventional subset isolation or flow gating strategies, this cell subset falls within the $T_N$ cell population. $T_{SCM}$ cells have been shown to contain higher levels of HIV-1 DNA compared to $T_N$ cells[340, 342, 344, 371]. However, the level of HIV-1 DNA in the $T_{SCM}$ cell population seems to be dependent on whether the individual initiated ART during

acute versus chronic infection[371]. These cells are distinct from both the $T_N$ and $T_{CM}$ cell subsets, as described in section 1.3.1, and can be differentiated from $T_N$ cells based on cell surface expression of CD95 and/or CD122[339]. $T_{SCM}$ cells constitute < 5% of the $T_N$ cell population in the peripheral blood[340-345], thus making this cell type difficult to study, particularly in HIV-1-infected individuals where the frequency of $T_N$ cells is significantly reduced. Much like the $T_{TM}$, $T_{EM}$, and $T_{TD}$ cells, the frequency of $T_{SCM}$ cells in the peripheral blood of HIV-1-infected individuals on suppressive ART remains relatively stable[340, 342]. In order to determine the contribution of the $T_{SCM}$ cells to the $T_N$ cell population, we depleted cells expressing CD95 from a fraction of the total CD45RA population, keeping both the CD45RA+CD95+ and the CD45RA+CD95- populations (see Fig. 5) for DNA quantification (Fig. 16). There were slightly higher levels of HIV-1 DNA in the CD45RA+CD95+ subset compared to the CD45RA+CD95- subset and the total $T_N$ compartment; however, this increase was not significant. In this study, $T_{SCM}$ cells do not seem to contribute significantly to the HIV-1 DNA reservoir within the total $T_N$ cell population.

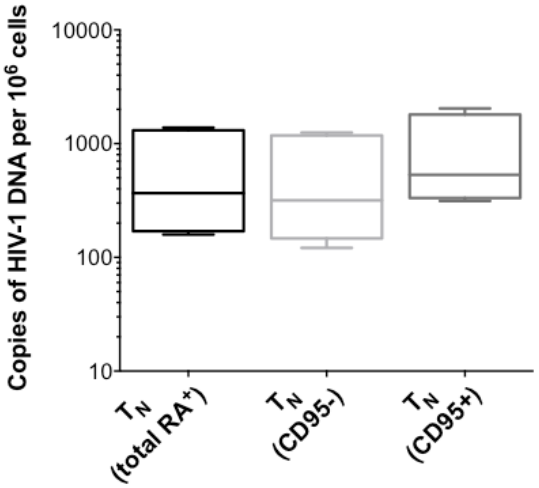


**Figure 16: Quantification of HIV-1 DNA in $T_N$ cell subsets.** CD4+ $T_N$ cell subsets were purified as described in figure 4. Total HIV-1 DNA was quantified in 1-2 million cells from donors 3-7 and normalized to total cell number assayed. Copies of HIV-1 DNA per $10^6$ cells are shown. No significant differences were found between any of the three cell subsets as determined by Mann-Whitney tests.

### 5.2.2 Latency reversal from $T_N$ and $T_{CM}$ cells following treatment with LRAs

In order to assess the ability of infected $T_N$ and $T_{CM}$ cells to produce virus, $T_N$ and $T_{CM}$ cells were stimulated with a panel of LRAs (Table 6) that have previously been shown or suggested to reactivate latent HIV-1 in rCD4 T cells. Our primary goal was to determine if virus could be produced from both cell types. As such, we largely chose LRAs that induce potent T cell activation in order to achieve a response. Additionally, we chose two HDACi that are in clinical evaluation as LRAs in hopes to reduce the HIV-1 latent reservoir[595, 656, 657]. The concentrations used and duration of RMD[513] and PNB[658] exposure were chosen based on predictions from previously determined clinical recommendations. Our secondary goal was to determine if virus production was equal between the two cell types when corrected for differences in the frequency of HIV-1 DNA.

**Table 6:** LRAs, proposed mechanism of latency reversal, and treatment conditions used in this study.

| Drug | Action | Concentration |
|---|---|---|
| **No drug** | Media control | - |
| **anti-CD3/CD28** | T cell activation (positive control) | 3 beads/cell |
| **PHA + IL-2** | Mitogen, cytokine | 10μg/mL, 100U/mL |
| **PMA + Ionomycin** | PKC agonist, $Ca^{2+}$ ionophore (DAG and IP3 analogs, respectively) | 5nM + 500μg/mL |
| **Prostratin** | PKC agonist | 5μM |
| **Panobinostat** | HDACi | 17.5nM, 30 min pulse |
| **Romidepsin** | HDACi | 50nM, 4 hr pulse |

In order to assess virus production, purified $T_N$ and $T_{CM}$ cells were stimulated with each of the LRAs in duplicate wells containing 1 million cells. Cells were cultured in the presence of EFV and RAL to prevent viral spread throughout the culture, as well as IL-2 to maintain cell viability. Seven days post-treatment, virion-associated HIV-1 RNA was quantified in the culture supernatant (Fig. 17A, see Materials and Methods for more details). Following treatment with LRAs, we found that $T_N$ and $T_{CM}$ cells produced similar levels of HIV-1 RNA averaged across all 7 donors (Fig. 17B). It has previously been demonstrated that HIV-1-infected cells from the peripheral blood of individuals on ART contain only a single integrated provirus[617, 659]. Therefore, we used copies of HIV-1 DNA as a surrogate marker for the number of HIV-1-infected cells. Interestingly, and in accordance with our previous findings using an in vitro primary cell model of HIV-1 latency (see chapter 4.0), when HIV-1 RNA levels were normalized

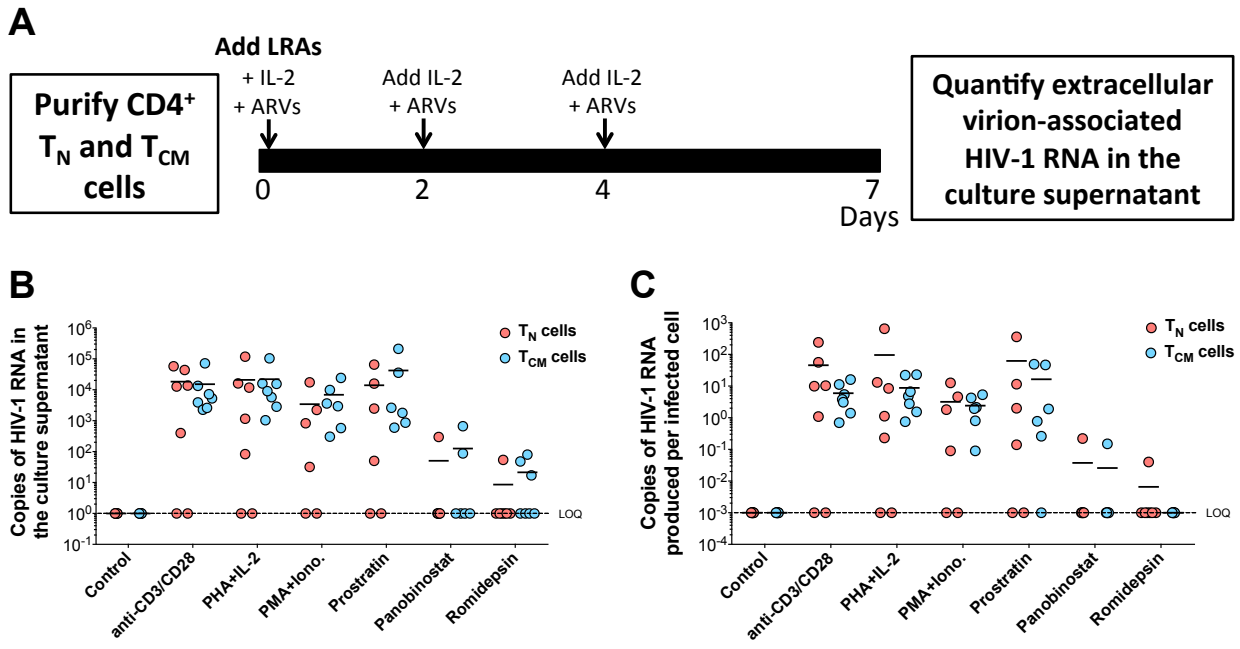


**Figure 17: Virus production following treatment with LRAs.** (**A**) Schematic representation of experimental approach. (**B**) Total virus production, measured as copies of HIV-1 RNA in the culture supernatant, following treatment with LRAs was quantified. The limit of quantification (LOQ) for this assay was 1 copy per reaction. (**C**) Copies of HIV-1 RNA in the culture supernatant were normalized to the number of infected cells in each cell subset. The calculated lower LOQ for HIV-1 RNA produced per infected cell was found to be 0.001. Each dot represents a unique donor. Horizontal bars represent the mean.

to the number of infected cells, there was more HIV-1 RNA produced per infected $T_N$ cell when treated with anti-CD3/CD28, PHA+IL-2, and prostratin, compared to infected $T_{CM}$ cell (Fig. 17C). Both RMD and PNB failed to increase virus production in both cell types in the majority of donors (Fig. 17C). This finding is consistent with previous studies demonstrating that HDACi, including RMD and PNB, are able to increase viral transcription but do not consistently increase virus production[482, 512, 534, 536].

These findings were remarkable because unlike in our primary cell model of HIV-1 latency, where significantly higher levels of infection can be achieved and virus production is observed in all donors, we only observed significant virus production from $T_N$ cells in 4 of 7 donors (Fig. 18). For example, two donors had no measurable virus production from $T_N$ cells following treatment with anti-CD3/CD28, while 1 donor had only very low levels of virus production (Table 7). In contrast, all 7 donors had high responses from their $T_{CM}$ cells (Table 7). Despite a lack of response in $T_N$ cells from 3 of 7 donors, total virus production was similar between $T_N$ and $T_{CM}$ cells (18,290 versus 15,135 copies/$10^6$ cells for $T_N$ and $T_{CM}$ cells,

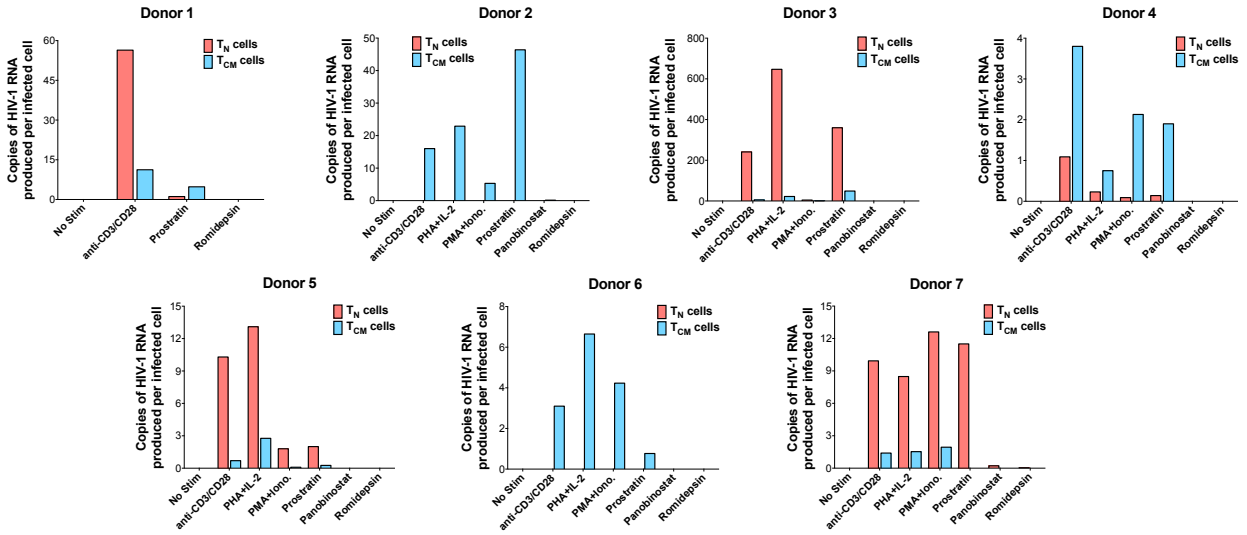


**Figure 18: Virus production from individual donors following treatment with LRAs.** Copies of HIV-1 RNA produced per infected $T_N$ and $T_{CM}$ cell are shown for each of the 7 donors.

respectively, Table 8). When corrected for differences in the number of infected cells between the two cell types, we found that $T_N$ cells produced 7.7 times more virus per infected cell than per infected $T_{CM}$ cell (Table 8). Although striking, due to variation between donors (Fig. 18), this difference is not significant.

**Table 7:** Copies of extracellular virion-associated HIV-1 RNA in the culture supernatant from each donor following treatment with anti-CD3/CD28.

| | **Donor 1** | **Donor 2** | **Donor 3** | **Donor 4** | **Donor 5** | **Donor 6** | **Donor 7** |
|---|---|---|---|---|---|---|---|
| **$T_N$ cells** | 57,663 | <1 | 43,600 | 397 | 12,670 | <1 | 13,700 |
| **$T_{CM}$ cells** | 13,400 | 71,350 | 3,890 | 5,245 | 2,260 | 7,210 | 2,590 |

**Table 8:** Average (mean) copies of HIV-1 RNA produced, and average (mean) copies of HIV-1 RNA produced normalized to HIV-1 DNA following treatment with anti-CD3/CD28.

| | **Mean copies of HIV-1 RNA produced per $10^6$ cells** | **Mean copies of HIV-1 RNA produced per HIV-1 DNA-containing cell** |
|---|---|---|
| **$T_N$ cells** | 18,290 | 45.5 |
| **$T_{CM}$ cells** | 15,135 | 5.91 |

In order to determine if the differences in virus production observed between $T_N$ and $T_{CM}$ cells were due to T cell activation or cell viability following treatment with LRAs, we measured the surface expression of the activation markers CD25, CD69, and HLA-DR on both cell types from donors 4-7 (Fig. 19). Additionally, we assessed cell viability by LIVE/DEAD staining in cells from donors 4-7 (Fig. 20). As expected, treatment with anti-CD3/CD28, PHA+IL-2, PMA+ionomycin, and prostratin induced T cell activation in both cell subsets. Only minor differences were noted in T cell activation following treatment with anti-CD3/CD28. No other significant differences were observed in the level of T cell activation measured on day 7 following treatment with LRAs between $T_N$ and $T_{CM}$ cells.

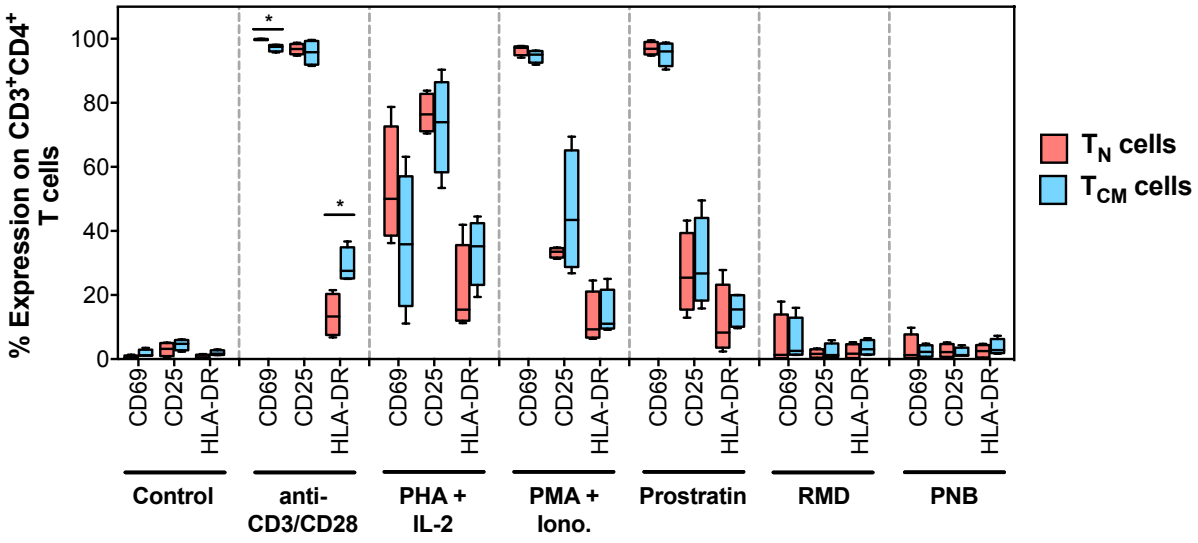


**Figure 19: Measure of T cell activation on $T_N$ and $T_{CM}$ cells following treatment with LRAs.** T cell activation was measured by cell surface expression of the T cell activation markers CD25, CD69, and HLA-DR 7 days post-treatment with LRAs. Each dot represents a unique donor. Data are shown from donors 4-7. Box and whisker plots represent the median (horizontal bars) and the range (whiskers). * $p < 0.05$ determined by a Mann-Whitney test.

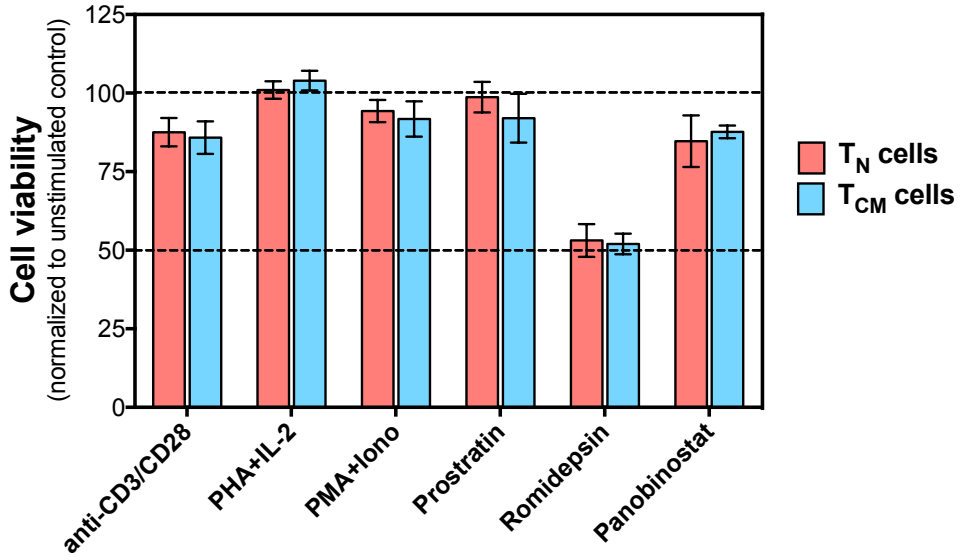


**Figure 20: Cell viability following treatment with LRAs.** Cell viability was measured by LIVE/DEAD staining 7 days post-treatment with LRAs in $T_N$ and $T_{CM}$ cells. Cell viability was normaized to the untreated control. Data are presented as the mean ± SEM. No significant differences were found for any LRA between the two cell types as measured by a Mann-Whitney test.

In addition to comparing virus production between $T_N$ and $T_{CM}$ cells, we also compared virus production from $T_N$ cells that either contained (RA+) or had been depleted of (-CD95) $T_{SCM}$ cells. $T_N$ cells from donors 5-7 were further purified, as described above (Fig. 16) to either contain only CD95+ cells or no CD95+ cells. Because the frequency of CD95+ cells within the $T_N$ cell population is so low, we were only able to conduct experiments using the CD45RA+CD95- subset in comparison to the total $T_N$ population. We found that similar levels of virus were produced from $T_N$ (RA+) cells, $T_N$ (-CD95) cells, and $T_{CM}$ cells, despite the fact that donor 6 had no measurable virus production from either $T_N$ (RA+) cells or $T_N$ (-CD95) cells (Fig. 21). Importantly, we found no significant difference in virus production between these two subsets when corrected for HIV-1 DNA, and $T_N$ (-CD95) cells from these three donors still produced as much, if not more virus per infected cell compared to the $T_{CM}$ cells in the absence of $T_{SCM}$ cells. This finding highlights that "true" $T_N$ cells that have been depleted of $T_{SCM}$ cells contribute significantly to virus production following latency reversal.

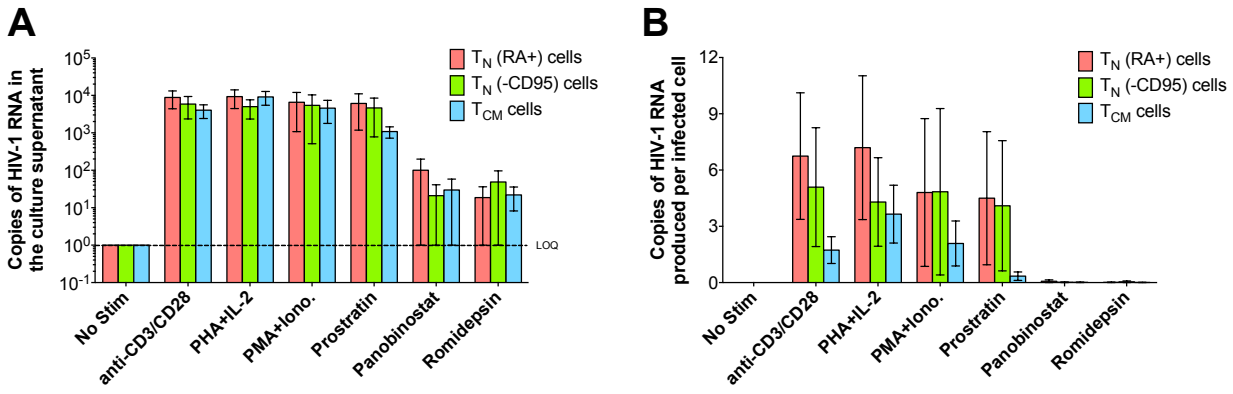


**Figure 21: Virus production from $T_N$ cells with and without CD95-expressing cells compared to $T_{CM}$ cells from donors 5-7 following treatment with LRAs.** (**A**) Total virus production was measured from $T_N$ cells either containing (RA+) or excluding (-CD95) CD95-expressing cells (see Materials and Methods for more details) from donors 5-7 and were compared to virus production from $T_{CM}$ cells from the same donors. (**B**) Copies of HIV-1 RNA in the culture supernatant from panel A were normalized to the number of infected cells in each cell subset. Data are presented as the mean ± SEM.

### 5.2.3 Measure of replication-competent HIV-1 in $T_N$ and $T_{CM}$ cells

In addition to quantifying HIV-1 RNA in the culture supernatant, we also measured replication-competent HIV-1 using the viral outgrowth assay from donors 4-7[613]. When comparing the frequency of cultured wells positive for replication-competent HIV-1, there was 4 times more replication-competent virus found in $T_{CM}$ cells compared to $T_N$ cells (Table 9). When we accounted for differences in HIV-1 DNA between $T_N$ and $T_{CM}$ cells, there is only 1.4 times more replication-competent virus found in $T_{CM}$ cells compared to $T_N$ cells (Table 9). This demonstrates that $T_N$ cells are not only able to produce virus following latency reversal, but a portion of the virus produced from these cells is replication-competent.

**Table 9:** Frequency of $T_N$ and $T_{CM}$ cell cultures yielding replication-competent HIV-1 from donors 4-7.

| | | | |
|---|---|---|---|
| **$T_N$ cells** | **Wells positive (%)** | **Fold difference ($T_{CM}/T_N$)** | **Fold difference corrected for HIV-1 DNA ($T_{CM}/T_N$)** |
| | 10/216 (4.63) | | |
| **$T_{CM}$ cells** | **Wells positive (%)** | 4 | 1.4 |
| | 40/216 (18.5) | | |

In addition to measuring replication-competent virus from $T_N$ and $T_{CM}$ cells, we were also able to measure replication competent virus from $T_N$ cells with and without CD95-expressing cells as done previously for total virus production using cells from donors 5-7 (see Fig. 21). Replication-competent virus was recovered from all three donors in both $T_N$ cell populations (Table 10). The frequency of replication-competent HIV-1 recovered from the two cell types was similar (4.44% vs. 5.56%), and when corrected for minor differences in HIV-1

DNA, there was slightly more replication-competent HIV-1 recovered from the $T_N$ (RA+) cell population than the $T_N$ (-CD95) cell subset (1.4 fold increase). In agreement with our latency reversal data (Fig. 21), "true" $T_N$ cells that have been depleted of $T_{SCM}$ cells contribute significantly to the production of replication-competent HIV-1 in comparison to the total $T_N$ population containing $T_{SCM}$ cells.

**Table 10:** Frequency of $T_N$ (RA+) and $T_N$ (-CD95) cell cultures yielding replication-competent HIV-1 from donors 5-7.

| | | | |
|---|---|---|---|
| **$T_N$ (RA+) cells** | **Wells positive (%)** | **Fold difference** [$T_N$ (RA+) cells / $T_N$ (-CD95) cells] | **Fold difference corrected for HIV-1 DNA** [$T_N$ (RA+) cells / $T_N$ (-CD95) cells] |
| | 8/180 (4.44) | | |
| **$T_N$ (-CD95) cells** | **Wells positive (%)** | 1.25 | 1.4 |
| | 10/180 (5.56) | | |

## 5.3 DISCUSSION

In this study, we evaluated the contribution on $T_N$ cells to the HIV-1 latent reservoir in HIV-1-infected long-term suppressed individuals. Evaluation of the efficacy of LRAs to reactivate latent HIV-1, either in terms of viral transcription or virus production, have largely been inconsistent between studies[471]. While certain LRAs prove effective in some models, they show no effect in others. This is likely, at least in part, due to the different cell types assayed between studies. Most in vitro primary cell models of HIV-latency focus on $T_{CM}$ cells or resting memory cells as a whole (discussed in section 1.4.3.3). In order to better understand the efficacy of LRAs across

studies, it is important to evaluate LRAs on an individual T cell subset basis. In one study, it was shown that treatment with anti-CD3/CD28 resulted in higher levels of virus production from all memory cell subsets compared to $T_N$ cells; however, when cells were stimulated with anti-CD3/CD28 + IL-7, the $T_N$ cells produced higher levels of HIV-1 RNA compared to the memory T cell subsets[600]. This finding emphasizes important differences between T cell subsets following latency reversal with different LRAs.

In this study, we found that following treatment with LRAs that induce T cell activation (Fig. 19), similar levels of virus were produced from $T_N$ and $T_{CM}$ cells averaged across 7 donors (Fig. 17B), despite there being little to no virus production from $T_N$ cells in 3 of 7 donors (Fig. 18). When we compared virus production between $T_N$ and $T_{CM}$ cells normalizing for HIV-1 infection, we found that as much, if not more, virus was produced per infected $T_N$ cell compared to infected $T_{CM}$ cell (Fig. 17C). These findings are consistent with our in vitro primary cell model data discussed in chapter 4.0.

One caveat to this is study is that among the 4 donors who showed more virus production from $T_N$ cells compared to $T_{CM}$ cells, we were unable to evaluate if this is due to (i) more latently infected $T_N$ cells producing virus than latently infected $T_{CM}$ cells, or (ii) more virus being produced per infected $T_N$ cell versus infected $T_{CM}$ cell. Given the extensive cell numbers required to perform such a detailed analysis[660], this evaluation was beyond the scope of this current study. However, the latency reversal data combined with the viral outgrowth data suggests that there is more virus being produced per infected $T_N$ cell.

Consistent with previous studies (as discussed in section 1.4.2), we found that $T_N$ cells contained significantly lower levels of HV-1 DNA than did $T_{CM}$ cells (Fig. 15). Despite containing significantly less HIV-1 DNA than $T_{CM}$ cells, $T_N$ cells produced more virus in 4 of 7

donors compared to $T_{CM}$ cells. This suggests that quantification of HIV-1 DNA alone may not predict the size of the inducible latent reservoir in different CD4+ T cell subsets. Furthermore, we demonstrated that even though $T_N$ cells produced more virus than $T_{CM}$ cells, there was more replication-competent virus recovered from $T_{CM}$ cells, even when taking into account the differences in HIV-1 DNA (Table 9). This suggests that the size of the inducible latent reservoir may not predict the size of the replication-competent reservoir in different CD4+ T cell subsets.

The data presented in this study highlight the significance of $T_N$ cells to the HIV-1 latent reservoir in patients on long-term ART and suggest that these cells may contribute significantly to viral rebound following treatment interruption or failure. As such, a greater attention should be given to this T cell subset when studying latency reversal strategies and they should not be excluded from such analyses.

# 6.0 NON-NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS REDUCE HIV-1 VIRUS PRODUCTION FROM LATENTLY INFECTED rCD4 T CELLS FOLLOWING LATENCY REVERSAL

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Author contribution: J.M.Z. performed all experiments and statistical analyses. N.S.C. and G.T. conceived of the study. J.M.Z. and N.S.C. drafted the manuscript. G.T. helped edit the manuscript.

## 6.1 INTRODUCTION

Latency reversal strategies have become a major area of research to reduce the size of the latent reservoir. It has been widely speculated that reduction or elimination of the latent reservoir in rCD4 T cells may lead to a functional or sterilizing cure[661, 662]. The "kick and kill" strategy[488, 489] has been the most widely utilized method to try and purge the latent reservoir, using a wide variety of LRAs. During latent HIV-1 infection, the integrated HIV-1 provirus remains transcriptionally silent in the absence of stimulation or cellular activation. However, upon cellular activation HIV-1 RNA is transcribed (latency is reversed) and virus is produced. Because this strategy induces virus production, experiments are always carried out in the presence of ART to prevent virus spread to new cells. In in vitro cell line and primary cell models, as few as one ARV can be used to prevent viral spread. This approach has been evaluated in several clinical studies[657], in which the ART regimen is dependent on each donor.

It is currently unknown if ARVs impact the efficacy of the "kick and kill" strategy. In this study, we determined whether different ARVs or ARV drug classes had an effect on the "kick and kill" approach using a primary cell model of HIV-1 latency described in chapter 3.0.

## 6.2 RESULTS

To address whether ARVs impacted the "kick" phenotype, we used a primary cell model that utilizes direct HIV-1 infection of highly purified rCD4 T cells to generate latently infected cells (Fig. 22A), as described previously[604]. The rCD4 T cells were infected with either X4-tropic HIV-$1_{LAI}$[605] or R5-tropic HIV-$1_{BaL}$[607]. Following the establishment of latency, the cells were

treated with one of several different ARVs from five distinct drug classes, including attachment inhibitors [MVC (CCR5 antagonist) or AMD3100 (CXCR4 antagonist)], NRTIs [lamivudine (3TC) or tenofovir (TFV)], NNRTIs [rilpivirine (RPV) or efavirenz (EFV)], an InSTI (RAL), and PIs [darunavir (DRV) or atazanavir (ATV)]. Following the addition of one of these ARVs, the latently HIV-1-infected rCD4 T cells were stimulated with anti-CD3/CD28 antibodies to reactivate latent HIV-1. Virus production was quantified by measuring extracellular virion-associated HIV-1 RNA in the culture supernatant, as described previously[160]. We found that equivalent amounts of R5-tropic (Fig. 22B) and X4-tropic (Fig. 22C) HIV-1 were generated from cells treated with attachment inhibitors, NRTIs, an INSTI, or PIs. In contrast, we observed a 10-fold or greater decrease in virus production from cells that had been treated with the NNRTIs, EFV or RPV (Fig. 22B, 22C). This decrease in HIV-1 production was not due to toxicity (Fig. 23A), or the NNRTI impacting global T cell activation (as assessed by CD25, CD69 or HLA-DR expression) in the absence (Fig. 23B) or presence (Fig. 23C) of anti-CD3/CD28 antibodies. Of note, more HIV-1 particle production was observed in the no ARV controls due to spread of the reactivated HIV-1 (Fig. 22B, 22C). The reduction in virus production following treatment of the latently HIV-1-infected rCD4 T cells with either EFV or RPV was dose-dependent for both the X4- (Fig. 22D) and R5-tropic (Fig. 22E) strains, with 50% inhibitory concentration (i.e., $EC_{50}$) in the low nanomolar range, which is equivalent to their $EC_{50}$ values for inhibition of reverse transcription[663].

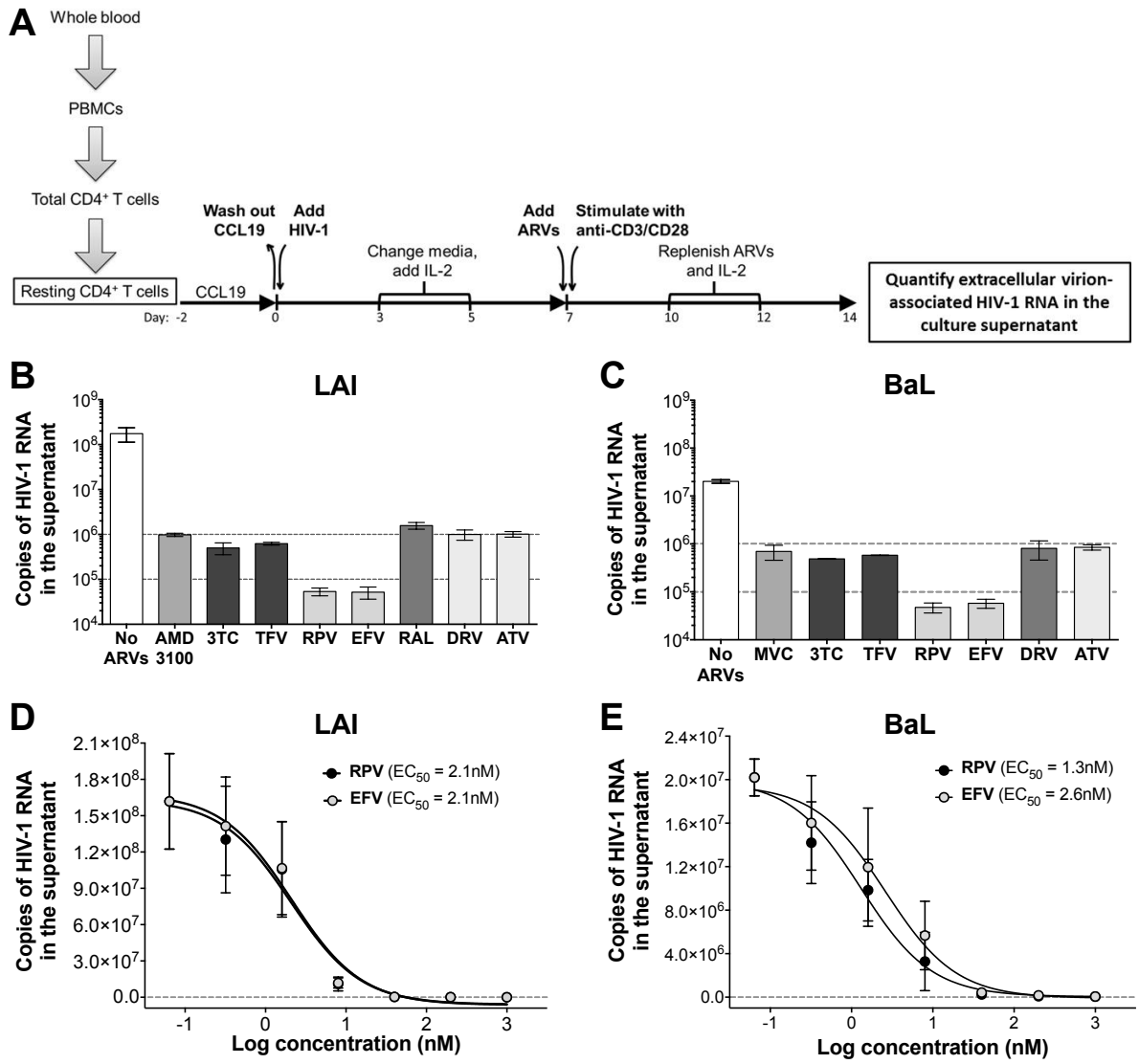



**Figure 22: NNRTIs reduce virus production following HIV-1 latency reversal in rCD4 T cells in vitro.** (**A**) Schematic of experimental approach. Copies of HIV-1 RNA in the culture supernatant are shown following treatment with anti-CD3/CD28 antibodies from cells infected with HIV-$1_{LAI}$ (**B**) and HIV-$1_{BaL}$ (**C**). Dose-dependent responses in virus production following treatment with RPV and EFV from cells infected with HIV-$1_{LAI}$ (**D**) and HIV-$1_{BaL}$ (**E**). $EC_{50}$ values were calculated using a three parameter nonlinear regression model in GraphPad Prism. Drug concentrations used: AMD3100 = 5μM; MVC = 1μM; 3TC = 50μM; TFV = 100μM; EFV = 1μM; RPV = 1μM; RAL = 500nM; DRV = 500nM; and ATV, 500nM. Error bars represent ± standard deviation. Data are from 2-4 independent experiments performed in duplicate.

Next, we asked whether NNRTIs decreased HIV-1 virus production by down-regulating HIV-1 gene transcription or protein translation. To address this question, we performed similar experiments in J89GFP cells, which are a Jurkat T-cell line that contains a stably integrated, full-length dual-tropic HIV-$1_{89.6}$ provirus with the EGFP reporter gene incorporated into the viral

genome[615]. The viral genome in these cells is transcriptionally silent. However, upon stimulation with PMA, viral transcription is activated and viral gene expression can be measured by EGFP expression, whereas virus production can be assessed by quantification of p24 antigen in the culture supernatant (Fig. 24). We found that neither the NNRTIs nor NRTIs reduced the levels of EGFP expression relative to the control (Fig. 24). However, consistent with the rCD4 T cell data, both EFV and RPV exhibited dose-dependent decreases in p24 production (Fig. 24).

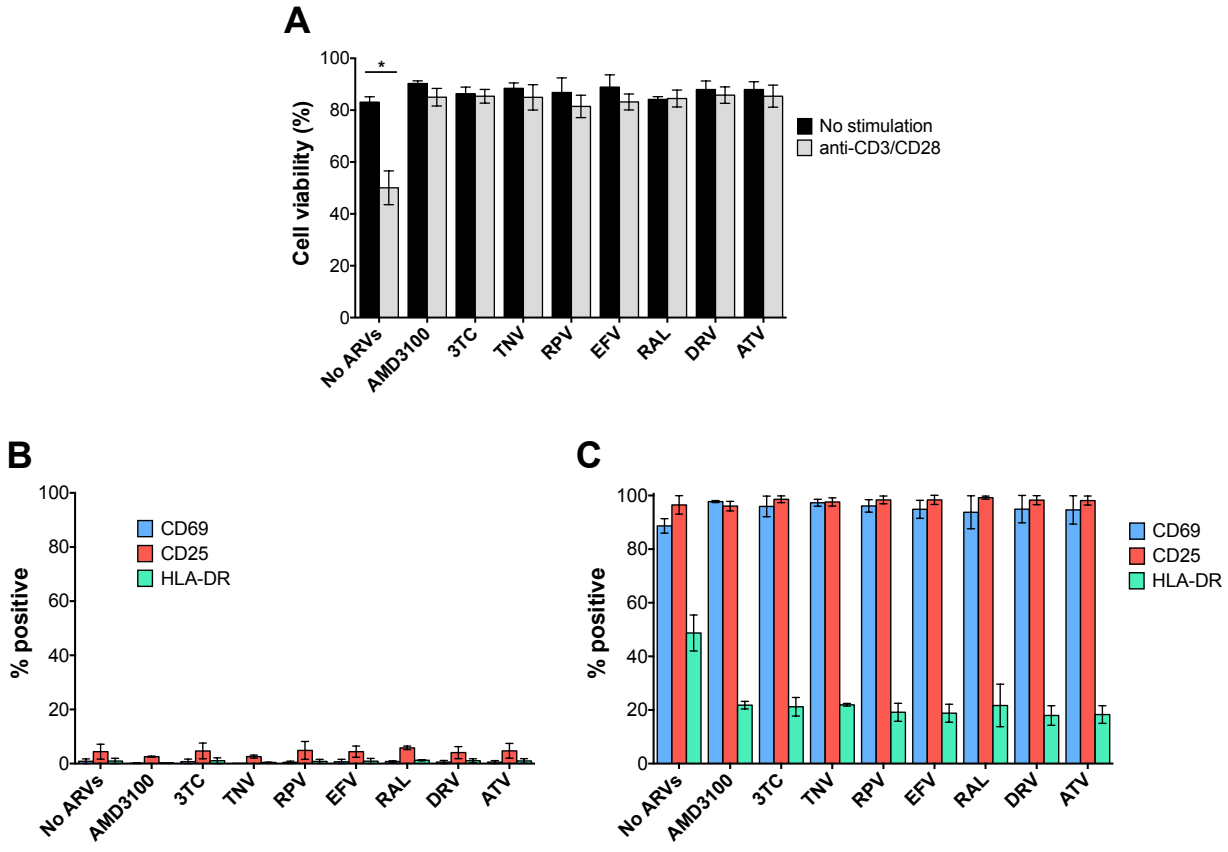



**Figure 23: T cell activation and cell viability following treatment of rCD4 T cells with different ARVs.** (**A**) Cell viability was measured by LIVE/DEAD staining and flow cytometry post-stimulation compared to unstimulated controls of cells infected with HIV-$1_{LAI}$. Surface expression of the T cell activation markers, CD25, CD69, and HLA-DR, were measured by flow cytometry seven days post-treatment with ARVs on cells infected with HIV-$1_{LAI}$ that were either (**B**) unstimulated or (**C**) stimulated with anti-CD3/CD28. * p value < 0.05 determined by a paired t test. Error bars represent ± standard deviation. Data are from 2-4 independent experiments performed in duplicate.

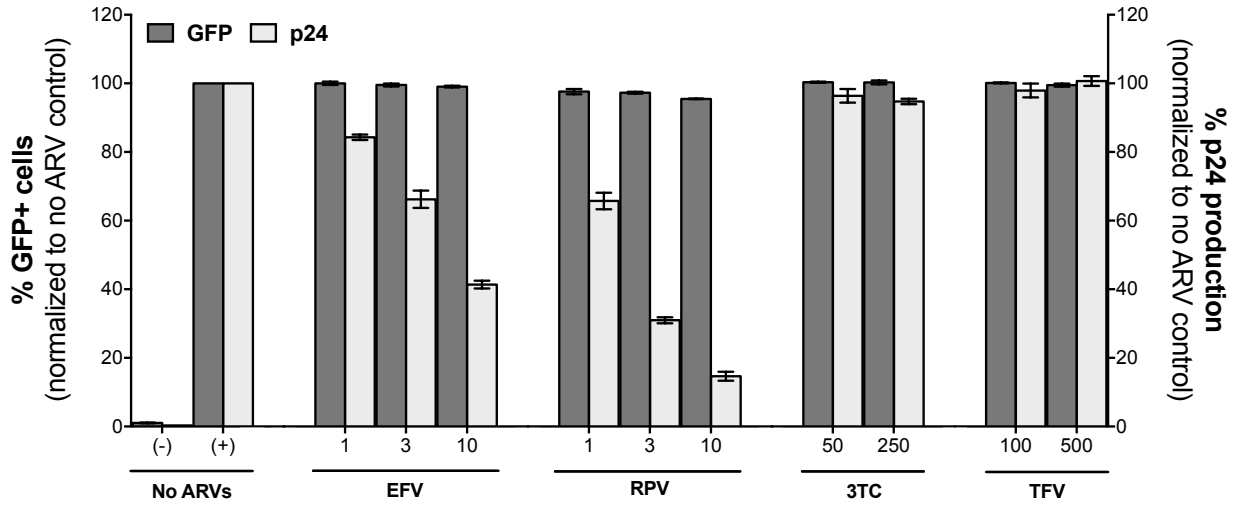



**Figure 24: NNRTI-reduced virus production is not due to decreases in HIV-1 transcription or protein translation.** In order to determine if the reduced virus production from cells treated with EFV or RPV was due to effects on transcription or translation, J89GFP cells were cultured in the absence or presence of different NNRTIs or NRTIs and were stimulated with PMA (30nM) for 48hrs. Following stimulation, the cells were analyzed by flow cytometry for cell viability, via LIVE/DEAD staining, and GFP expression. Virus production was quantified by p24 in the culture supernatant. (-) and (+) refer to without and with PMA stimulation in the absence of any ARVs as a control. All concentrations shown are in micromolar. For both GFP expression and p24 production, samples were normalized to the no ARV (+) PMA control, which was set at 100%.

Collectively, these data reveal that the NNRTIs EFV and RPV significantly attenuate the "kick" of latent HIV-1 from rCD4 T cells by inhibiting release of HIV-1 virus particles. This finding is consistent with our prior work which demonstrated that potent NNRTIs impact the late stages of HIV-1 replication[602], which leads to a decrease in virus production from HIV-1-transfected 293T or HeLa cells[603, 664]. Interestingly, in the HeLa and 293T cells micromolar concentrations of EFV were required to see a significant reduction in virus production[603, 664]. In contrast, the concentrations of EFV or RPV required to decrease virus production from rCD4 T cells was in the nanomolar range (Fig. 22D, 22E), and is significantly lower than the peak plasma concentrations following a single oral dose in humans [1.6-9.1μM for EFV[665]; 0.39-0.53μM for RPV[666]]. This suggests that NNRTIs may decrease virus production in HIV-1-

infected individuals using NNRTI-containing regimens enrolled in intervention studies aimed at eradicating the latent HIV-1 reservoir.

To assess whether ARVs impacted the "kill" phenotype due to the cytopathic effect of the reactivated virus, we quantified the number of HIV-1-infected cells for both HIV-$1_{LAI}$ (Fig. 25A) and HIV-$1_{BaL}$ (Fig. 25B) before and after exposure to the anti-CD/CD28 antibodies by qPCR analysis of total HIV-1 DNA. HIV-1 DNA was normalized to the total number of cells assayed (assessed by qPCR amplification of the *CCR5* gene [608]), as described previously[235]. We observed significant decreases in the total number of HIV-1-infected cells treated with each of the ARVs following stimulation with anti-CD3/CD28 when compared to the unstimulated controls (Fig. 25A, 25B). We still observed death of infected cells treated with EFV and RPV, even in the absence of virus production (Fig. 25A, 25B). This is consistent with previous findings that NNRTIs increase intracellular processing of Gag and Gag-Pol[602, 603, 664]. This NNRTI-induced intracellular processing of Gag/Gag-pol leads to premature protease activation, which is cytotoxic and can result in cell death[667, 668].

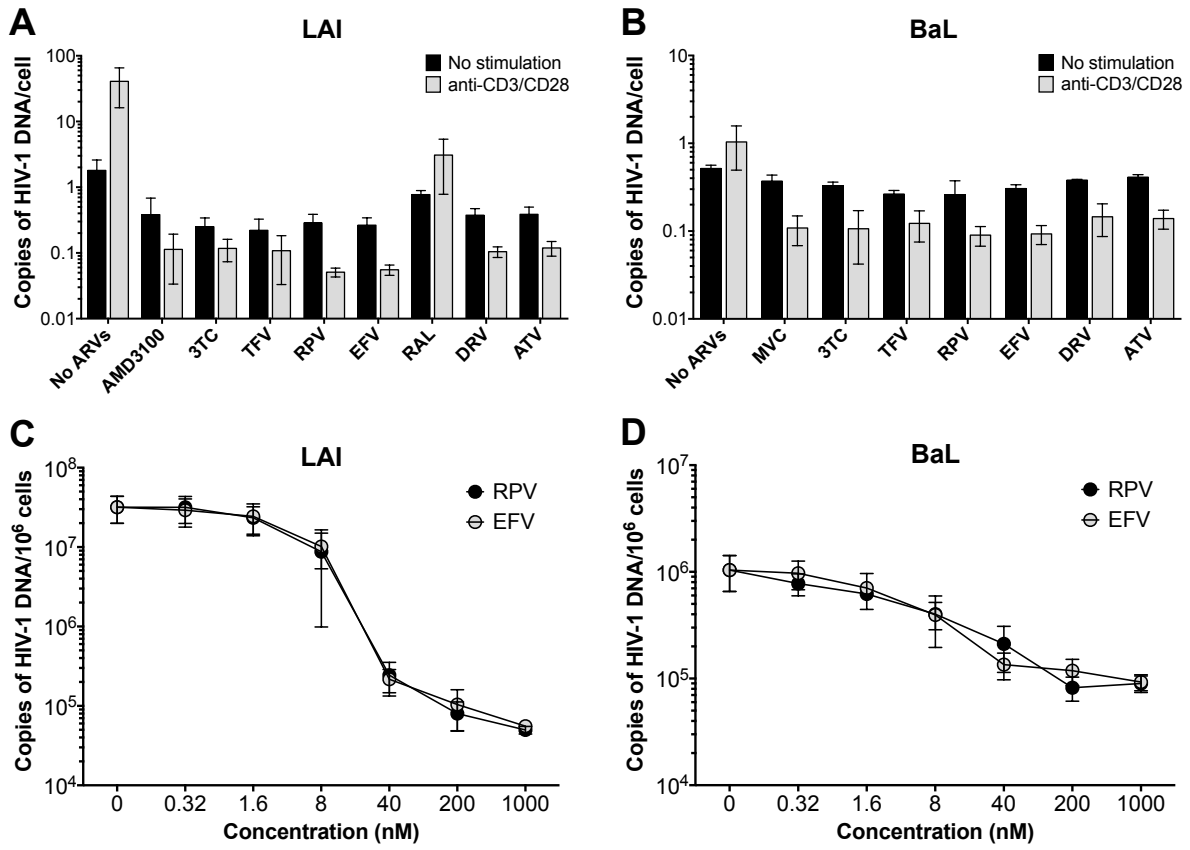



**Figure 25: Changes in HIV-1 DNA following latency reversal when cells are treated with different ARVs.** Copies of HIV-1 DNA per cell were quantified in both stimulated and unstimulated cells infected with (**A**) HIV-$1_{LAI}$ (**B**) or HIV-$1_{BaL}$. Dose-dependent decrease in HIV-1 infection following treatment with RPV and EFV of cells infected with (**C**) HIV-$1_{LAI}$ and (**D**) HIV-$1_{BaL}$. Error bars represent ± standard deviation. Data are from 2-4 independent experiments performed in duplicate.

## 6.3 DISCUSSION

In summary, our data demonstrate that NNRTIs reduce HIV-1 production from latently infected rCD4 T cells compared to other classes or ARVs. These findings have significant implications for HIV-1 eradication studies. For example, ex vivo studies that use NNRTIs to prevent virus spread, or rCD4 T cells from donors on NNRTI containing regimens, should be cautiously interpreted. Furthermore, it may be more difficult to observe an increase in plasma viral load

following the administration of a LRA to an HIV-1-infected participant on a NNRTI based therapy. Finally, our data suggest that there may be a therapeutic application for NNRTIs in helping to eliminate cells expressing HIV-1 Gag and Gag-Pol proteins, although additional studies are warranted to further explore this possibility.

# 7.0 CONCLUSIONS

Through the work presented here from AIMs 1 and 2, we have exposed the significance of $T_N$ cells to the overall HIV-1 latent reservoir. Through the modification of a primary cell model of HIV-1 latency, which allowed for the evaluation of $T_N$ cells while maintaining the integrity of freshly isolated rCD4 T cell subsets, we were able to recapitulate the differences in infection frequency between $T_N$ and $T_{CM}$ cells seen in HIV-1-infected individuals. This provided a useful tool to evaluate both the establishment and reversal of HIV-1 latency. Throughout the development of this model, we demonstrated that our culture conditions as well as in vitro HIV-1 infection did not affect the resting cell phenotype, assessed by no change in T cell activation or proliferation. Importantly and significantly, this is the first in vitro primary cell model reported to generate direct infection in purified $T_N$ cells.

With this model, we evaluated the similarities and differences of the "kick and kill" strategy on $T_N$ and $T_{CM}$ cells following latency reversal. As expected, we found more total virus production from $T_{CM}$ cells compared to $T_N$ cells following latency reversal. However, $T_N$ cells produced as much, if not more, virus than $T_{CM}$ cells when normalized for differences in HIV-1 infection (HIV-1 DNA). This result was unexpected and demonstrated that $T_N$ cells may be a more important contributor to the latent viral reservoir than previously thought.

Another unexpected finding from this study was that CCL19-mediated direct infection of $T_N$ and $T_{CM}$ cells did not occur through alleviation of the two known major restriction factors to

HIV-1 infection of rCD4 T cells, the actin cytoskeleton and SAMHD1. These data suggest that there are additional blocks to HIV-1 infection of rCD4 T cells, which have not yet been identified and which are alleviated by CCL19 treatment. Further evaluation of the mechanisms of CCL19-mediated enhancement of HIV-1 infection is greatly needed, both to identify novel restriction factors in rCD4 T cells, as well as to gain a better biological understanding of direct infection of these cells.

As with any cell model, there is always the question of in vivo relevance. While we could not directly evaluate the in vivo contribution of $T_N$ cells following latency reversal, we could validate our findings from AIM 1 ex vivo from cells obtained from HIV-1-infected individuals on long-term ART. Consistent with our findings using our primary cell model, we found that $T_N$ cells produced more virus than $T_{CM}$ cells in 4 out of 7 donors, even when uncorrected for differences in infection frequency. In this study, we were also able to measure the frequency of replication-competent virus between $T_N$ and $T_{CM}$ cells in a subset of donors and found that the level of replication-competent virus between these two cell types was similar when normalized for differences in infection. Interestingly, we did not find more replication-competent virus in $T_N$ cells from donors who had higher levels of virus production compared to $T_{CM}$ cells. Although based on a limited sample size, these findings demonstrate that the size of the HIV-1 DNA reservoir is not necessarily predictive of the size of the inducible reservoir, and the size of the inducible reservoir is not necessarily predictive of the size of the infectious reservoir.

The field of HIV-1 latency is heavily focused on quantitative measures to try and predict subsequent outcomes. While PCR-based measures are extremely sensitive and are necessary to quantify the low-level frequency of latently infected cells in HIV-1 infected individuals, these assays over-predict the size of the inducible latent reservoir because they do not distinguish

between infectious versus non-infectious nucleic acid[441]. Conversely, measures of the infectious viral reservoir have been shown to underestimate the size of the latent reservoir, likely due to many contributing factors[601]. One factor is that not all replication-competent proviruses become reactivated following a single low-dose stimulation with PHA[601]. It has previously been shown that a single stimulation is only able to induce a fraction of total replication-competent proviruses, while a second stimulation is able to induce an additional fraction of proviruses[601]. However, in this study, not all replication-competent proviruses were inducible, even after multiple stimulations. The authors of this study concluded that the size of the inducible latent reservoir is at least 60-fold greater than that predicted by the QVOA. A second major factor of underestimating the reservoir size is that this assay requires a weekly addition of CD8-depleted, PHA-activated, allogeneic feeder cells to propagate infectious virus to detectable levels[613]. This relies on both the susceptibility of the feeder cells to HIV-1 infection, and the functionality of these cells to support robust, productive infection. The vast differences in donor susceptibility to HIV-1 infection, as well as the differences in virus production following latency reversal in "kick and kill" studies suggests that the use of allogeneic feeder cells will affect the outgrowth of infectious virus following co-culture. The findings presented in AIM 2 of this thesis are in line with these previous works and warrant caution when interpreting inferred correlations based on quantitative analyses.

Moving forward, it is clear that evaluation of all CD4+ T cell subsets that constitute the latent viral reservoir is necessary. These different CD4+ T cell subsets have phenotypic and physiological differences that likely contribute to the establishment and reversal of HIV-1 latency in each cell type in vivo. Evaluations of these differences are in their infancy and are not well understood. Once proviral latency is established, maintenance of latency is highly regulated

and multifaceted. As described previously in section 1.4.4, it is becoming clear that individual LRAs are inefficient at reversing latent HIV-1, while combinations of LRAs are proving to be more effective. There are likely different mechanisms involved in the maintenance of latent HIV-1 in the different CD4+ T cell subsets that may need to be targeted differently in order to achieve a maximal response. Gaining a better understanding of both the establishment and maintenance of HIV-1 latency in different CD4+ T cell subsets will help guide more targeted assessment of LRAs and provide critical insight into LRA combinations that may be needed to reduce or eliminate the latent reservoir.

In AIM 3, we evaluated the effect of different ARVs and ARV classes on the “kick and kill” approach. Importantly, this strategy is always employed in the presence of ART, either as ART in vivo or with as little as a single ARV in vitro. How these ARVs may influence responses to LRAs has not previously been evaluated. We found that the NNRTIs EFV and RPV significantly inhibited virus production from latently infected cells following treatment with anti-CD3/CD28. Consistent with previous reports, we found that the restriction imposed by these NNRTIs is at the level of virus production and not transcription or translation. This finding suggests that in vitro evaluation of LRAs should not be conducted in the presence of NNRTIs, as they may mask responses induced by the LRAs if the assay readout is an extracellular virion measure. The in vivo implications of these findings are currently unclear.

While the “kick and kill” approach has been evaluated as a possible mechanism to eradicate the latent viral reservoir, studies thus far have largely evaluated the “kick”, with little emphasis on the "kill”. Latency reversal strategies using a single agent have thus far proven to be ineffective. LRAs that induce T cell activation or proliferation are toxic and LRAs that modestly induce viral transcription seem to have little to no effect on reducing the size of the latent

reservoir. Additional evaluation of the mechanisms that maintain HIV-1 latency in different T cell subsets could provide useful insights into how to specifically target these cells for viral reactivation. Further evaluations on the reversal of HIV-1 latency in different T cell subsets could help provide information about which drug combinations may need to be used to achieve maximal reactivation.

In practicality, it seems unlikely that the "kick and kill" approach will not work in HIV-1-infected individuals on ART to significantly reduce or eliminate the latent reservoir. The biggest challenge with this approach is that all current LRAs are not specific for HIV-1 but instead target cellular factors. This raises major concerns regarding off target effects that could be toxic or lethal. Additionally, the only LRAs that have been found to significantly increase virus production and/or reduce the size of the latent reservoir ex vivo induce T cell activation, which would be an undesirable and toxic outcome to in vivo treatment.

An alternative approach to achieve a functional cure may be through the therapeutic establishment of permanent latency. In 2012, it was shown that an analog of the natural steroid alkaloid Cortistatin A, didehydro-Cortistatin A (dCA), inhibited Tat-mediated transcription and diminished spontaneous virus production from CD4+ T cells purified from HIV-1-infected individuals on ART[669]. A follow-up study revealed that dCA induced long-term control of HIV-1 transcription in CD4+ T cells purified from HIV-1-infected individuals on ART, even after dCA had been removed, and after stimulation with anti-CD3/CD28 or prostratin[670]. This led the authors to conclude that dCA established a state of permanent latency, possibly through epigenetic modification or repression of the HIV-1 promoter[670]. This could represent a novel class of ARVs that could induce a state of permanent latency and establish a functional cure. To date, the drug triptolide is the only Tat inhibitor or inhibitor of HIV-1 transcription to reach

clinical trials[671, 672]. Clinical evaluation of dCA would determine if permanent latency could be established in vivo and if long-term control of viral replication could be maintained off ART.

# 8.0 FUTURE DIRECTIONS

The data presented in this thesis shed light on novel characteristics of the HIV-1 latent reservoir that demonstrated that $T_N$ cells are a major contributing cell type to this reservoir in terms of viral rebound following latency reversal. While many key findings were identified through this work, several additional questions were raised along the way. A few key questions raised will be discussed below with suggested experiments on how to address them.

## 8.1 HOW DOES CCL19 PROMOTE INFECTION OF rCD4 T CELLS?

Using our primary cell model of HIV-1 latency, we showed that CCL19 enhances infection of both rCD4 $T_N$ and $T_{CM}$ cells, albeit to different degrees. A previous report suggested that CCL19 promoted infection of rCD4 T cells by enhancing actin dynamics and F-actin density[456]. Our data presented in chapter 4.0 demonstrated that while inhibition of F-actin assembly does inhibit HIV-1 infection (Fig. 9), CCL19 does not alleviate the restriction to HIV-1 infection in rCD4 T cells imposed by the actin cytoskeleton (Fig. 10). A publication from the same group came out during the writing of this thesis that now suggests that CCL19-mediated enhancement of HIV-1 infection is driven by phosphatidylinositol-3-kinase (PI3K)-mediated activation of NF-κB[673]. Importantly, in the case of F-actin, changes were only seen at a maximum of 30 minutes post-CCL19 treatment[456], while for NF-κB activation, changes were only measured up to 15 minutes

post-CCL19 treatment[673]. Importantly, in these two studies, cells were infected between 24-72 hours post-CCL19 treatment[456, 673], long after the proposed mechanisms of enhancement were measured. NF-κB activation is most frequently associated with increased HIV-1 transcription; however, it has been shown in various T cell lines that HIV-1 integration and the establishment of HIV-1 latency is dependent on low-levels of NF-κB[674, 675].

Early on in our studies to determine how CCL19 enhances HIV-1 infection of rCD4 T cells, we also suspected involvement of PI3K signaling given that many chemokines induce signaling through PI3K pathways to modulate cell migration, cell survival, and increase cellular functionality[676]. In addition, CCL19 has been shown to increase PI3K activation in many different types of cancer[677-682]. To test our hypothesis, we treated rCD4 T cells with the PI3K inhibitor, PI-103, at various concentrations prior to the addition of CCL19. Following CCL19 treatment for two days, cells were infected with HIV-$1_{LAI}$. Seven days post-infection, HIV-1 DNA was quantified in both the PI-103-treated and the CCL19 only cells. We did not see inhibition of HIV-1 infection following PI-103 treatment and did not see a dose response to treatment (Fig. 26), and therefore decided not to move forward with this approach.

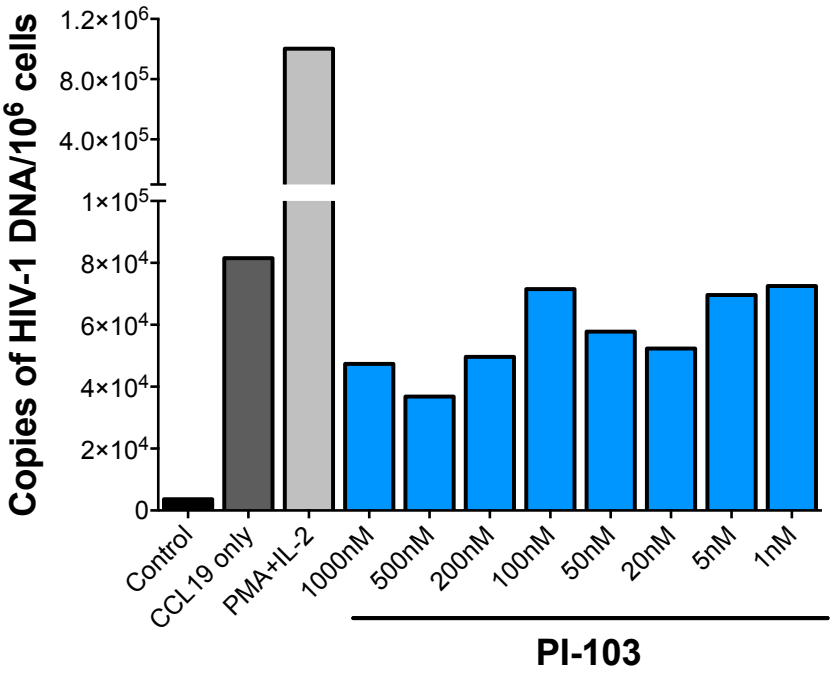



**Figure 26: Inhibition of the PI3K pathway does not inhibit HIV-$1_{LAI}$ infection of CCL19-treated rCD4 T cells.** rCD4 T cells were either treated with varying concentrations of the PI3K inhibitor, PI-103, or were left untreated for 6 hrs prior to treatment with CCL19 for 48 hours. Following treatment with CCL19, cells were infected with HIV-$1_{LAI}$ and remained in culture for 7 days. Seven days post-infection, HIV-1 DNA was quantified and normalized to cell number. Cells left untreated or stimulated with PMA + IL-2 (10nM, 50U/mL) for 48 hours prior to infected were used as controls. Preliminary data, N = 1.

The data published, both by us and other groups, on the mechanism(s) of CCL19-enhanced HIV-1 infection are an incomplete picture of what CCL19 might be doing to prime rCD4 T cells for HIV-1 infection. This is largely due to the approaches taken, which rely on published data or hypotheses as to known restriction mechanisms to HIV-1 infection in rCD4 T cells or known pathways that can modulate HIV-1 infection. CCL19 may enhance HIV-1 infection of rCD4 T cells in a manner that has never been explored before or act through an unknown restriction factor.

An alternative approach to identify cellular pathways that are modulated following treatment with CCL19 is through the use of the Phospho Explorer Antibody Array developed by

Full Moon Biosystems. This technology measures the phosphorylation or dephosphorylation of over 400 different proteins using 1318 site-specific antibodies that target over 30 signaling pathways. Instead of relying on guesswork, we have performed two preliminary experiments using the Phospho Explorer Antibody Array technology in which we compared the changes in phosphorylation following CCL19 treatment versus no treatment for 6 hours (Fig. 27A) or 24 hours (Fig. 27B). Although the data are only from a single experiment at each time point, it highlights two important findings. One is that there is little to no overlap in the proteins that are significantly increased or decreased in phosphorylation following CCL19 treatment at either time point between $T_N$ and $T_{CM}$ cells. This suggests that either CCL19 enhances HIV-1 infection of rCD4 T cells via a pathway not identified in this screen, or that CCL19 enhances HIV-1 infection of $T_N$ and $T_{CM}$ cells through different mechanisms, which may help explain why there is a greater enhancement of HIV-1 infection in $T_{CM}$ cells compared to $T_N$ cells. Secondly, these data highlight the importance of the timing of the experiments as there was also little to no overlap in the significantly phosphorylated or dephosphorylated proteins following CCL19 treatment in $T_N$ and $T_{CM}$ cells at the two different time points. In addition to the proteins that are identified in Figure 24 that were either significantly phosphorylated or dephosphorylated following CCL19 treatment, there were many additional proteins that had changes in phosphorylation that neared significance (data not shown).

In these initial screens, we also found no significant changes in either cell type in the phosphorylation of cortactin, cofilin, filamin A, or LIMK1, which are all cofactors that mediate actin polarization and actin dynamics[683-689], in addition to actin itself (data not shown). This further supports our conclusion that CCL19-mediated enhancement of HIV-1 infection does not depend on changes in the actin cytoskeleton. Interestingly, and in partial agreement with the

recently published paper[673], these screens also found a significant increase in two heterodimeric forms of NF-κB at both 6 and 24 hours post-CCL19 treatment for $T_N$ cells but not for $T_{CM}$ cells (Fig. 27). NF-κB is a family of transcription factors made up of several different heterodimeric or homodimeric proteins that can become activated through many different extracellular stimuli to regulate a wide range of genes[690-692]. Therefore, further investigation is needed to understand the significance of increased NF-κB activation following CCL19 treatment and how this may affect HIV-1 infection of rCD4 T cells.

Additional experiments should be conducted with cells from multiple donors to gain a better understanding of the differences and similarities in CCL19-mediated signaling between $T_N$ and $T_{CM}$ cells. We would also like to assess phosphorylation/dephosphorylation events at different time points because it has previously been published that CCL19-mediated enhancement of HIV-1 infection can occur anywhere between 3-72 hours post-CCL19 treatment[456]. We preliminarily chose to look 6 and 24 hours post-CCL19 treatment as a starting point with the plan to look at both an earlier and later time point. To further evaluate the importance of proteins that are significantly phosphorylated/dephosphorylated following CCL19 treatment, Full Moon Biosystems has 33 additional phosphorylation profile arrays targeting specific pathways for a more in-depth analysis of each pathways. They also have 9 protein expression profile arrays that could be used in addition to the phospho arrays to identify increased or decreased protein expression following CCL19-mediated signaling events. These combined approaches would give a more comprehensive and non-biased overview of cellular changes that occur following CCL19 treatment. The most difficult challenge will be to ascertain which cellular change(s) is responsible for CCL19-mediated enhancement of HIV-1 infection in rCD4 T cells.

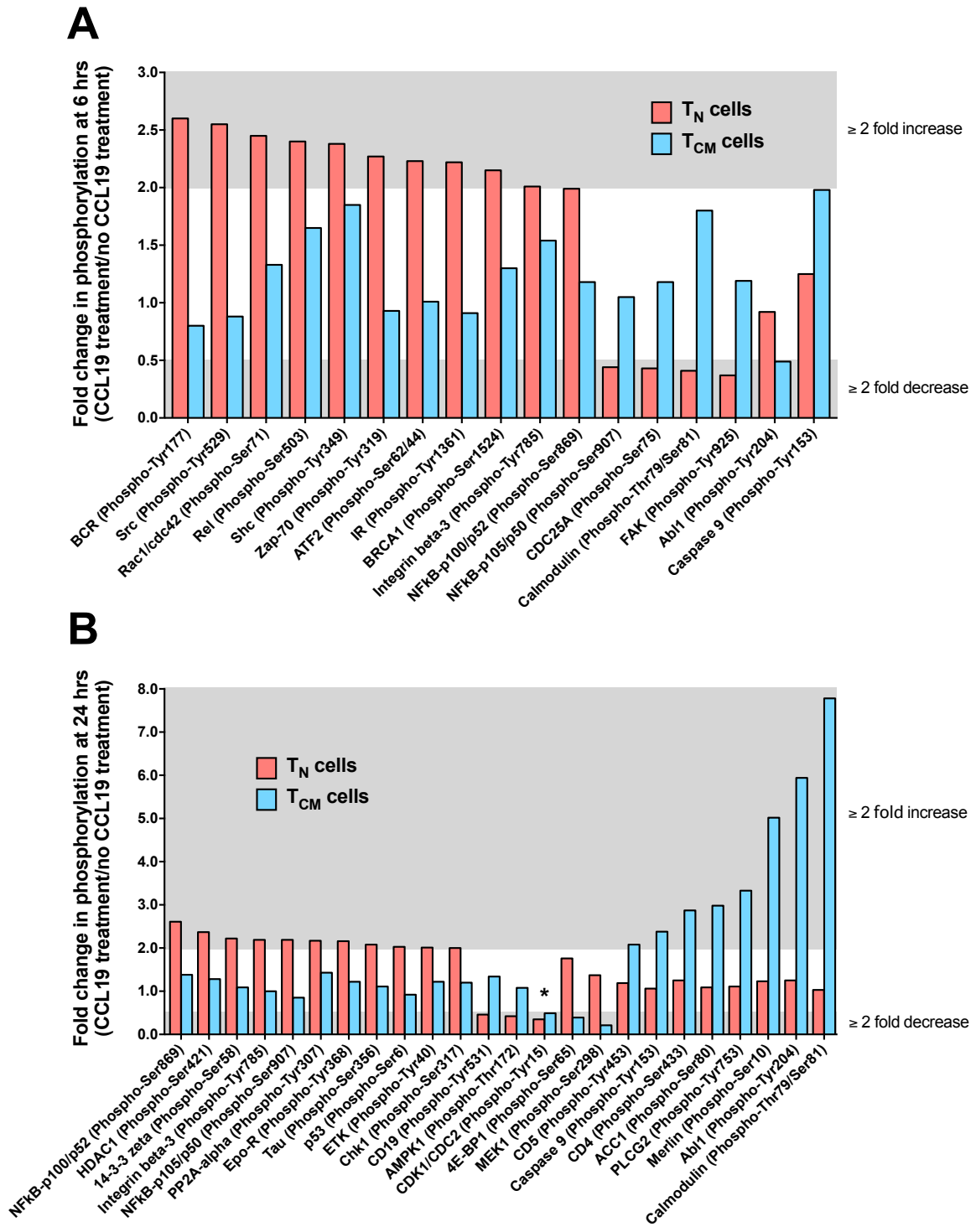



**Figure 27: Significant increase or decrease in phosphorylation of specific proteins following CCL19 treatment identified by Phospho Explorer Antibody Array imaging.** Significant changes (± 2-fold increase or decrease compared to no CCL19 control) in the phosphorylation of specific proteins following treatment with CCL19 for 6 hrs (**A**) or 24 hrs (**B**) identified by the phospho explorer antibody array from Full Moon Biosystems, are shown for both $T_N$ and $T_{CM}$ cells. Gray shading highlights the areas of significance. * = significant change in phosphorylation for both $T_N$ and $T_{CM}$ cells. N = 1 for both A and B.

## 8.2 IS THERE MORE VIRUS BEING PRODUCED PER INFECTED CELL OR MORE INFECTED CELLS ABLE TO PRODUCE VIRUS?

One major question that we have yet to answer in either our primary cell model of HIV-1 latency or in HIV-1-infected cells purified from long-term suppressed individuals, is whether $T_N$ cells are able to produce more virus per infected cell or if more infected $T_N$ cells are able to produce virus than infected $T_{CM}$ cell. $T_N$ cells are often overlooked or considered as an insignificant cell type to the HIV-1 latent reservoir based on the consistently lower frequency of HIV-1 DNA when compared to the memory cell subsets. However, in the context of latency reversal and virus production following latency reversal, $T_N$ cells were found to produce as much, if not more, virus than $T_{CM}$ cells when corrected for differences in the frequency of HIV-1 infection in the majority of individuals evaluated. This raises a critical biological question surrounding where virus comes from following viral rebound after treatment cessation or failure, in addition to latency reversal strategies.

One way to address this question would be to perform limiting dilution cultures of both $T_N$ and $T_{CM}$ cells purified from HIV-1-infected individuals on suppressive ART in replicate cultures. Following maximal stimulation of each well with anti-CD3/CD28 antibodies to induce virus production from latently infected cells, virus production can be measured in the culture supernatant by qRT-PCR. With the limiting dilution culture method, Poisson statistics can be used to determine the probability that virion production in a given well came from a single provirus. This would allow us to quantitatively determine the amount of virus produced from individually infected $T_N$ and $T_{CM}$ cells. If individual proviruses from $T_N$ cells produce fewer or similar levels of virus compared to $T_{CM}$ cells, this would suggest that more infected $T_N$ cells are able to produce virus compared to $T_{CM}$ cells following stimulation. Alternatively, if individual

proviruses from $T_N$ cells produce more virus than $T_{CM}$ cells, then we would know that there is more virus being produced per infected $T_N$ cell compared to infected $T_{CM}$ cell.

This method has been previously described and was used to determine virion production from single inducible proviruses following T cell activation[660]. In this paper, it was found that virion production from individual proviruses could vary as much as 100,000-fold using rCD4 T cells purified from HIV-1-infected individuals on ART. These findings further highlight not only the dramatic differences between individually infected cells but also between donors. Breaking the latent reservoir in rCD4 T cells down into individual subsets could help explain some of these differences if certain cell subsets (such as the $T_N$ cells) are able to produce more virions per inducible provirus in comparison to other cell subsets (such as the $T_{CM}$ cells). Although it was beyond the scope of this previous study, it would have been informative to look at virion production in each donor individually instead of combined and then also measure the frequency of each T cell subset constituting the rCD4 T cells to determine if there were any relationships between cell frequency and virion production.

## 8.3 IS THE BLOCK IN VIRUS PRODUCTION AT THE LEVEL OF TRANSCRIPTION, TRANSLATION, OR BUDDING?

If it turns out that there is more virus being produced per infected $T_N$ cell compared to $T_{CM}$ cell, it is important to understand if this is due to differences in transcription, translation, or viral budding post-stimulation. Elucidating the mechanistic differences in virus production between different cell types may help guide more directed approaches to latency reversal strategies. At least one study has found that there was no relationship between levels of unspliced cellular

HIV-1 RNA transcription and the level of virion production following treatment with SAHA[693], which suggests that there are likely post-transcriptional blocks following latency reversal that have not yet been described. If there are additional post-transcriptional blocks that prevent virus production following latency reversal, identifying new targets may help provide more potent LRA combinations to efficiently reactivate latent HIV-1 and reduce/eliminate the latent reservoir.

There are several different approaches that could be taken to quantify latency reversal at several different stages in the HIV-1 replication cycle post-stimulation. To measure differences in the initial "kick" in transcription following treatment, intracellular levels of full-length HIV-1 mRNA could be quantified by qRT-PCR. To measure translation of viral proteins, the intracellular p24 capsid protein is the most frequently measured by either an ELISA or antibody labeling and flow cytometry. Using an ELISA to measure p24 protein levels would give an actual quantitative value of p24 (usually in nanograms) in a given number of cells, whereas flow cytometry would only be able to provide relative differences in p24, measured by the MFI, between $T_N$ and $T_{CM}$ cells. Either approach would be able to address differences in protein production between $T_N$ and $T_{CM}$ cells.

If there were no observed differences between $T_N$ and $T_{CM}$ cells in terms of viral transcription or translation following LRA stimulation, it would be important to determine if there were defects in either viral assemble or budding from the plasma membrane. To measure differences between viral proteins in the cytoplasm and viral proteins at the plasma membrane in an assembling virion, equilibrium flotation centrifugation can be used to separate membrane bound from unbound viral proteins using cell suspensions[694]. To determine if viral proteins are being retained in the cell and not leading to the production of virions, western blot analysis can

be used to measure viral protein levels between cell lysates and virus produced post-stimulation with an LRA. This will determine if assembling virions are somehow retained on the plasma membrane and are unable to bud from the cell.

Using these techniques, there are three potential outcomes that would distinguish between differences in HIV-1 assembly versus budding: i) there could be equal levels of virion assembly and virion production between $T_N$ and $T_{CM}$ cells, suggesting no difference in these processes between these cell types, ii) there could be higher levels of assembling virions in $T_N$ cells but lower levels of virion production compared to $T_{CM}$ cells, suggesting a block at the level of virion budding in $T_N$ cells, and iii) there could be higher levels of assembling virions in $T_{CM}$ cells but lower levels of virion production compared to $T_N$ cells, suggesting a block at the level of virion budding in $T_{CM}$ cells. These data could identify novel restriction factors or post-translation blocks to HIV-1 replication following latency reversal that could aid in the development of novel ARV targets.

## 8.4 NON-RESTING $T_N$ OR MEMORY CD4+ T CELL SUBSETS THAT CONTRIBUTE TO THE LATENT RESERVOIR AND VIRAL PERSISTENCE

HIV-1 latency has almost exclusively been identified and studied in rCD4 T cells. This may be due to the fact that rCD4 T cells are easy to study on a population level. rCD4 T cells largely only contain HIV-1-infected cells that are in a latent state, therefore, when studying these cells as a population, HIV-1-producing cells do not have to be distinguished from the non-producing cells. Furthermore, rCD4 T cells constitute the majority of CD4+ T cells in the peripheral blood and are therefore easy to obtain from HIV-1-infected individuals. However, in HIV-1-infected

individuals on long-term ART, in which viral replication is fully suppressed, integrated HIV-1 DNA has been found in a wide range of other CD4+ cell types, both in the periphery and in tissues, suggesting that these cells likely contain latent proviruses (reviewed in [599, 695]).

In addition to the $T_N$ and memory CD4+ T cell subsets discussed throughout this thesis, additional CD4+ T cell subsets have been implicated as reservoirs for latent HIV-1 in infected individuals on long-term ART, such as the T follicular helper cells ($T_{FH}$), T helper 17 cells ($T_H17$), T regulatory cells ($T_{Reg}$), and even activated or partially activated CD4+ T cells[599, 695-697]. These cell types are largely confined to tissues, making them harder to evaluate for HIV-1 infection. Additionally, it has now been shown, using an in vitro primary cell model, that HIV-1 latency can be established in both resting and activated CD4+ T cells[697]. This is consistent with the finding that HIV-1 DNA can readily be detected in peripheral activated or total CD4+ T cells in HIV-1-infected individuals on ART, in the absence of viral replication.

Although integrated HIV-1 DNA has been measured in all of these CD4+ T cell subsets in HIV-1-infected individuals on ART, there is limited information on their actual contribution to the latent viral reservoir or viral persistence. Both peripherally-isolated, HIV-1-infected $T_{FH}$[698] and $T_{Reg}$[699] cells from patients on ART have been shown to persist for several years by longitudinal analyses. It was also shown that latently infected $T_{Reg}$ cells could be activated to produce virus following stimulation with PHA + IL-2[699]. These data strongly suggest that peripheral $T_{FH}$ and $T_{Reg}$ cells contribute to the latent viral reservoir in HIV-1-infected individuals on ART. However, tissue reservoirs have not yet been sufficiently evaluated. Additional techniques and ex vivo analysis is required to fully interrogate HIV-1 persistence in tissue-derived reservoirs. Sequence analysis of integrated proviruses coupled with measures of viral infectivity could help evaluate the contribution of these tissue-derived reservoirs to the total pool

of replication-competent, inducible proviruses. A further analysis of HIV-1 latency and persistence in individual CD4+ T cell subsets will help give a more complete picture of the composition of the latent reservoir in HIV-1-infected individuals on ART and may help identify more directed approaches to reduce or eliminate latently infected cells.

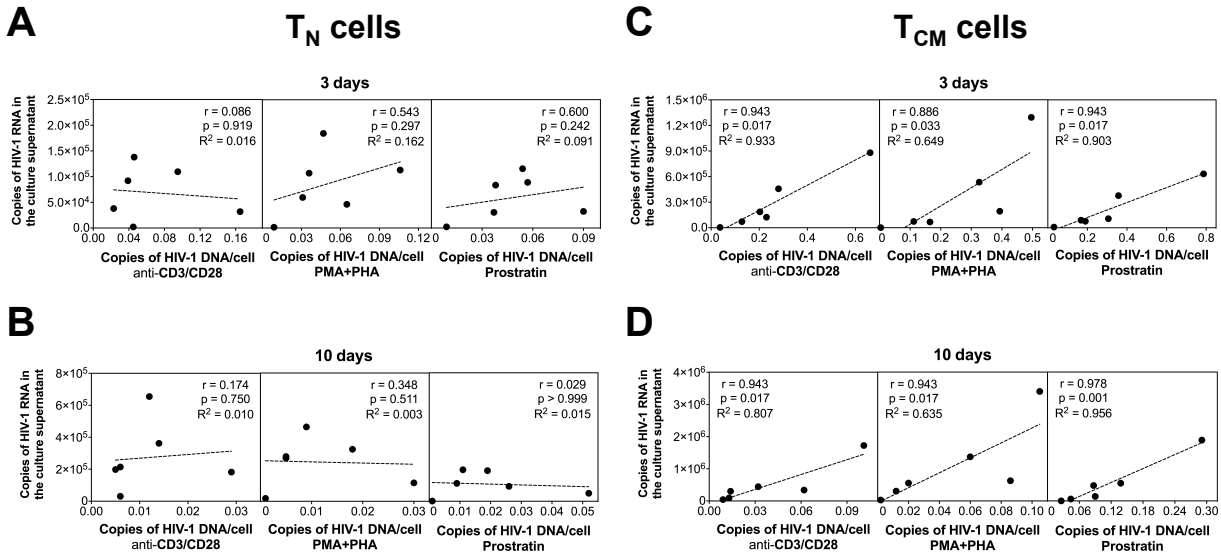
# APPENDIX A

## A.1 SUPPLEMENTAL DATA FROM CHAPTER 4.0

**Supplementary Table 1:** P values for comparison of PHA-activated CD4+ T cell integration site distribution to $T_N$ and $T_{CM}$ cells, and to the MRC dataset.

| | Sample | MRC | Activated CD4+ T cells[a] | CD4+ $T_N$ cells |
|---|---|---|---|---|
| Within RefSeq genes[b] | **Activated CD4+ T cells[a]** | $< 2.2 \times 10^{-308}$ | - | - |
| | **CD4+ $T_N$ cells** | $3.45 \times 10^{-106}$ | 0.344 | - |
| | **CD4+ $T_{CM}$ cells** | $< 2.2 \times 10^{-308}$ | 0.003 | 0.022 |
| Within 5kb (± 2.5kb) of a transcriptional start site[b] | **Activated CD4+ T cells[a]** | $1.68 \times 10^{-09}$ | - | - |
| | **CD4+ $T_N$ cells** | 0.85 | 0.54 | - |
| | **CD4+ $T_{CM}$ cells** | 0.72 | 0.24 | 1.00 |
| Within 5kb (± 2.5kb) of a CpG island[b] | **Activated CD4+ T cells[a]** | $1.03 \times 10^{-21}$ | - | - |
| | **CD4+ $T_N$ cells** | 0.64 | 0.41 | - |
| | **CD4+ $T_{CM}$ cells** | 0.84 | 0.02 | 0.76 |
| Average gene density within 1Mb (± 0.5Mb) of integration sites[c] | **Activated CD4+ T cells[a]** | $< 2.2 \times 10^{-308}$ | - | - |
| | **CD4+ $T_N$ cells** | $1.66 \times 10^{-101}$ | $2.3 \times 10^{-04}$ | - |
| | **CD4+ $T_{CM}$ cells** | $< 2.2 \times 10^{-308}$ | 0.05 | 0.02 |

Actual counts of sites within RefSeq genes and relative to CpG islands and TSS's, as well as regional gene densities, are shown in Table 3. [a]PHA-activated CD4+ T cells, [b]P values calculated by Fisher's exact test, [c]P values calculated by Wilcoxon rank sum test.




**Supplementary Figure 1: Correlation analyses between level of infection and virus production post-stimulation from $T_N$ and $T_{CM}$ cells latently infected with HIV-$1_{LAI}$.** Correlation analysis of $T_N$ cells three (**A**) and ten (**B**) days post-stimulation with anti-CD3/CD28 microbeads, PMA+PHA, and prostratin. Correlation analysis of $T_{CM}$ cells three (**C**) and ten (**D**) days post-stimulation with anti-CD3/CD28 microbeads, PMA+PHA, and prostratin. P value determined by Spearman correlation analysis. Spearman correlation coefficients (r) are shown. Each dot represents a different donor. Dotted lines represent linear regression curve.

## APPENDIX B

## LIST OF ABBREVIATIONS

**2-LTR:** 2-long terminal repeat-containing circles

**3TC:** Lamivudine

**Ab:** Antibody

**Ag:** Antigen

**AIDS:** Acquired immunodeficiency syndrome

**Akt:** Protein kinase B

**AML:** Acute myeloid leukemia

**AP-1:** Activator protein-1

**ART:** Antiretroviral therapy

**ARV:** Antiretroviral

**ASCT:** Allogeneic stem cell transplantation

**ATI:** Analytic treatment interruption

**ATV:** Atazanavir

**bp:** base pair

**CA:** Capsid

**Cas:** CRISPR-associated nuclease

**CDK9:** Cyclin-dependent kinase 9

**cDNA:** Complementary DNA

**CRISPR:** Clustered regularly interspersed palindromic repeats

**CSF:** cerebral spinal fluid

**CTD:** C-terminal domain

**DAPI:** 4',6-diamidino-2-phenylindole

**dCA:** Didehydro-Cortistatin A

**DNA:** Deoxyribonucleic acid

**DNMTi:** DNA methyltransferase inhibitor

**dNTP:** Deoxynucleoside triphosphate

**dNTPase:** Deoxynucleoside triphosphate triphosphohydrolase

**DRV:** Darunavir

**DSIF:** DRB-sensitivity inducing factor

**DZNep:** 3-deazaneplanocin A

**EC:** Elite controller

**EFV:** Efavirenz

**ELISA:** Enzyme-linked immunosorbent assay

**ESCRT:** Endosomal sorting complex required for transport

**FACS:** Fluorescence-activated cell sorting

**FBS:** Fetal bovine serum

**FDA:** Food and Drug Administration

**GALT:** Gut-associated lymphoid tissue

**GVHD:** Graft-versus-host disease

**HDACi:** Histone deacetylase inhibitor

**HIV-1:** Human immunodeficiency virus type-1

**HLA:** Human leukocyte antigen

**HMBA:** Hexamethylbisacetamide

**HMTi:** Histone methyltransferase inhibitor

**HSCT:** Hematopoietic stem cell transplant

**IL-2:** Interleukin-2

**IN:** Integrase

**InSTI:** Integrase strand transfer inhibitor

**IUPM:** Infectious units per million cells

**kB:** Kilobases

**LOD:** Limit of detection

**LRA:** Latency reversing agent

**LTNP:** Long-term non-progressor

**LTR:** Long-terminal repeat

**MA:** Matrix

**MDDCs:** Monocyte-derived dendritic cells

**MFI:** Mean fluorescence intensity

**MOI:** Multiplicity of infection

**MoM:** Myeloid only mice

**NC:** Nucleocapsid

**NELF:** Negative transcription elongation factor

**NFAT:** Nuclear factor of activated T cells

**NHP:** Non-human primate

**NNRTI:** Non-nucleoside reverse transcriptase inhibitor

**NPC:** Nuclear pore complex

**NRTI:** Nucleoside reverse transcriptase inhibitor

**PBS:** Phosphate buffered saline

**PFA:** Paraformaldehyde

**PHA:** Phytohemagglutinin

**PI:** Protease inhibitor

**PI3K:** Phosphatidylinositol-3-kinase

**PKC:** Protein kinase C

**PMA:** Phorbol-12-myristate 13-acetate

**PNAd:** Peripheral-node addressin

**PNB:** Panobinostat

**Pro:** Protease

**P-TEFb:** Positive transcription elongation factor b

**PTEN:** Phosphatase and tensin homolog

**qPCR:** Quantitative polymerase chain reaction

**qRT-PCR:** Quantitative reverse transcriptase polymerase chain reaction

**QVOA:** Quantitative viral outgrowth assay

**pDC:** Plasmacytoid dendritic cells

**RAL:** Raltegravir

**rCD4:** Resting CD4+ T cell

**RIC:** Reduced-intensity chemotherapy

**RMD:** Romidepsin

**RNA:** Ribonucleic acid

**RNAi:** RNA interference

**RPV:** Rilpivirine

**RRE:** Rev response element

**RT:** Reverse transcriptase

**SAHA:** Suberoylanilide hydroxamic acid

**SAMHD1:** Sterile alpha motif and histidine/aspartic acid domain-containing protein 1

**SCID:** Severe combined immunodeficiency

**SD:** Standard deviation

**SEM:** Standard error of the mean

**shRNA:** Short hairpin RNA

**siRNA:** Small interfering RNA

**SIV:** Simian immunodeficiency virus

**snRNP:** Small nuclear ribonucleoprotein

**TALENS:** Transcription activator-like effector nucleases

**TAR:** Transactivation response element

**Tat:** Trans-activator of transcription

**TBI:** Total body irradiation

**$T_{CM}$:** Central memory CD4+ T cells

**$T_{EM}$:** Effector memory CD4+ T cells

**$T_{FH}$:** T follicular helper cells

**TGF-β:** Tissue growth factor-beta

**$T_H17$:** T helper 17 cells

**ToM:** T cell only mice

**$T_N$:** Naïve CD4+ T cells

**TNF:** Tumor necrosis factor

**$T_{Reg}$:** Regulatory T cells

**TSA:** Trichostatin A

**$T_{SCM}$:** Stem cell-like memory CD4+ T cells

**TSSs:** Transcription start sites

**$T_{TD}$:** Terminally differentiated CD4+ T cells

**$T_{TM}$:** Transitional memory CD4+ T cells

**VNP:** Viremic non-progressor

**ZFN:** Zinc-finger nuclease

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